

A QUALITATIVE AND QUANTITATIVE DETERMINATION
OF THE AMINO ACIDS AND AROMA SUBSTANCES,
ESPECIALLY ESTERS, ALDEHYDES AND
KETONES IN MUSTS AND WINES.

by

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Thesis submitted for the Degree of
DOCTOR OF SCIENCE in AGRICULTURE
at the University of Stellenbosch.

The crest of the University of Stellenbosch is centered behind the text. It features a shield with various symbols, topped with a crown and a banner. The Latin motto "Pectora robustant cultus recti" is inscribed on a ribbon below the shield.

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November 1958.

A C K N O W L E D G E M E N T S .

To my promotor PROF. DR. KURT HENNIG,
Geisenheim/Rhein, I tender my sincerest thanks for expert
guidance offered and keen interest shown in this investi-
gation.

Towards the KO-OPERATIEWE WIJNBOUWERS
VERENIGING VAN ZUID-AFRIKA BEPERKT I feel deeply indebted
for a scholarship enabling me to carry out this work during
a two-year period commencing August, 1956.

I also wish to express my gratitude towards
THE UNION DEPARTMENT OF AGRICULTURE for the generous
gesture of allowing me to remain in its full employ during
the said period.

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I N T R O D U C T I O N .

The methods which have been employed up till the present time for the estimation of individual wine components were chiefly within what might be described as the bounds of Macrochemistry.

The modern microchemical methods of analysis, by means of Paper Chromatography, have made it possible to establish the presence and in many instances the concentration of rare components.

The aforesaid macrochemical methods are employed chiefly for the determination of individual substances and their quantitative estimation within a certain group of allied substances, e.g. in the total titratable acid group, the lactic, malic, tartaric and volatile acids.

The work of Hennig and Burkhardt¹⁾ on the tannins and polyphenolic substances of wine has shown that although the total acid content of a wine is not represented by the aforesaid substances alone, but also includes other compounds, e.g. chlorogenic and p.-coumaric acids. These lastmentioned acids appear in wine in such minute quantities that their presence can in no way whatsoever be reflected by the ordinary macrochemical titration of the total titratable acids. Mainly due to these facts lack of knowledge as to their presence and function must be ascribed. Such compounds which through their molecular structure are partly to be designated as acids and partly as polyphenols and tannins, are of importance in the development of a wine.

This work may be regarded as an attempt, through the application of microchemical paperchromatographical methods to estimate certain micro substances, i.e. amino acids, esters, aldehydes and ketones in musts and wines.

Some amino acids are indispensable during the fermentation and development of a wine and also perform a definite function in the development of flavour and bouquet.

The esters, alcohols, aldehydes and ketones are the most important components of the wine bouquet. Through a pentane extraction process, Hennig and Villforth²⁾ succeeded in extracting from a wine its bouquet-forming components. Their experiments proved conclusively that the extracted substances are of the utmost importance in rendering to a wine its bouquet

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or aroma, i.e. they are quality determining factors. The pentane extraction had practically no influence whatever on the macrochemical analysis of the treated wine. From the point of view of taste, a sweetish-sour liquid, which could perhaps rightly be described as the "mortal remains" of the wine, was retained.

Smell and taste are in the generally accepted sense not easily separated. When a wine is tasted, it is primarily the aroma that is being established.

Kirchner³⁾ contends that taste has four components, i.e. salty, bitter, sour and sweet. Salty and sour differ from bitter and sweet in that the latter taste components are the manifestation of an ionic reaction. The best example of a salty taste is offered by common table salt. Although the salty taste is the result of an ionic reaction, the explanation is not altogether simple. If the salty taste were due to the chlorine ions, then the addition of hydrochloric acid should increase the saltiness, and reversely, if it were due to the sodium ions, then it should follow that all sodium compounds should have a similar salty taste. Thus it can only be assumed that the sodium and chlorine ions together and through their particular molecular arrangement, determine the salty taste.

Fabian and Blum⁴⁾ during the course of work on the compensating and competitive taste influences of various foodstuffs, found that the addition of acetic, lactic, malic and tartaric acids, increase the salty taste of table salt.

Harvey⁵⁾ carried out work on the relationship between total acidity, pH-value and taste, and found that the acid taste is not purely a function of the H-ion-concentration, but it is also partly governed by the anions.

The bitter astringent taste is associated with the alkaloids, many aromatic glucosides and tannins. Quinine and piperine are examples of such alkaloids. Hesperine is a glucoside found in orange peel, whilst the astringent taste associated with unripe apples is ascribed to a bountiful supply of tannins.

A sweet taste may originate from many and varied substances of widely different chemical origin, e.g. the polyhydroxy acids, saccharin and the amino acids. Täufel⁶⁾ points out that on the formation of a molecule of maltose from two molecules of

glucose.....

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glucose, a decline in sweetness is registered, while on the other hand, when one molecule of fructose combines with one molecule of glucose, the "sweetness" is increased.

In spite of extensive work in this field, it has, as yet, not been possible to establish a precise relationship between chemical constitution on the one hand, and taste and smell on the other hand. This still remains a problem and serves here merely to indicate the difficult problems in this particular field. Barral and Ranc⁷⁾ found that stereo-isomeric compounds could be of widely different tastes, e.g. d-phenylamine and d-histidine are sweet, whilst their corresponding l-compounds are bitter. The chemical properties of optical isomers are identical, thus it is logical to conclude that chemical constitution and taste are not always bound in a fixed relationship.

The personal expression in judging a particular aroma must also be taken into account. A completely unknown substance may receive a widely differing reception when a panel consisting of persons selected at random is called upon to judge it according to taste and smell. The aroma of a wine is determined by its volatile constituents, most of which are not ascertainable by the generally applied physical and chemical methods of analysis. As knowledge as to the identification and estimation of such substances is seriously lagging behind, and as a modest endeavour to stimulate further work in this field, this study was undertaken.

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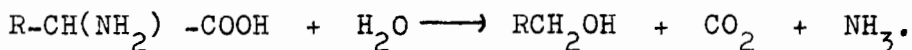
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A QUALITATIVE AND QUANTITATIVE DETERMINATION
OF THE AMINO ACIDS IN MUSTS AND WINES.

THE ORIGIN OF THE AMINO ACIDS - FACTS AND THEORIES.

It has long been known that the amino acids play an important part during the fermentation and development of a wine. They are of importance to the yeast and the origin of the fusel oils is partly associated with the amino acids. They are also partly responsible for the aroma substance formation and are generally believed to be of major importance in the biological degradation of the acids in wines.

Pasteur already recognised that nitrogenous compounds are necessary for the development of yeasts. Asparagine has long been regarded as an effective foodstuff for the yeasts and Behrens¹⁾ and Pringsheim²⁾ ascertained that yeasts require nitrogenous compounds during the fermentation of the sugars. Such substances should generally possess the following grouping: $R-CH(NH_2)CO-$. Pringsheim and Ehrlich³⁾ investigated the formation of the higher alcohols and contended that the higher alcohols originate in the sugars via the amino acids of the yeast cell.



An alcohol possessing one carbon atom less than the original amino acid is thus formed. The resulting nitrogen is either directly utilised by the yeasts or eventually figures as ammonia in wine. Thus is yielded:-

From Valine	Isobutyl alcohol
Leucine	Isoamyl alcohol
Isoleucine	Optically active Amyl alcohol
α -Aminobutyric acid	n-Propyl alcohol.

These alcohols are grouped together under the common heading "Fusel Oils".

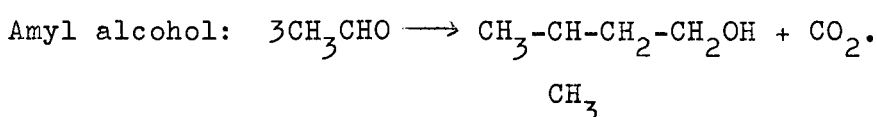
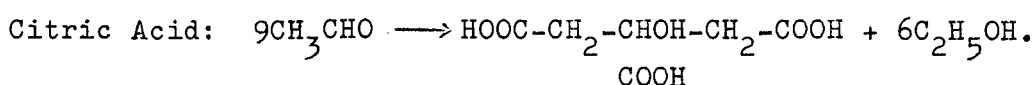
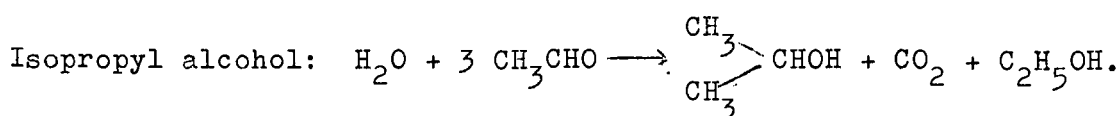
The pungent smell often regarded as the major aroma-yielding component in young wines, or wines resulting from musts of low sugar content, are regarded by Ehrlich⁴⁾ and Hennig and Ohske⁵⁾ as being due to the presence of esters of amyl alcohol. Villforth and Schmidt^{6 & 7)} during the course of their work on the higher alcohols in wine, determined all the higher alcohols together and designated them "Fusel Oils". They also found that the presence of organic nitrogenous compounds increased fusel oil formation, while the converse held in the presence of inorganic nitrogenous compounds such as NH_3 -compounds. Proteins resulting from decaying yeast cells

have.....

have a positive influence on the production of higher alcohols. The addition of sugar also reflected itself in a higher production of fusel oils.

Castor⁸⁾ also occupied himself with this problem. Prior to fermentation the amounts of three different amino acids were ascertained and afterwards compared with produced amounts of fusel oil. He contended that fusel oil formation is governed by two major factors, i.e. the nature of the amino acids present and the "nitrogen management" of the yeasts present. As contended by Villforth⁹⁾ he also holds that NH_4 ions restrict the formation of higher alcohols, up to 30% less being registered. The formation of higher alcohols from amino acids is a reaction requiring energy. This energy is supplied by the sugar fermentation.

Here the work of Genevois and Lafon¹⁰⁾ must be cited. Through the application of marked acetic acid, Genevois¹¹⁾ succeeded in proving the generally presumed transformation of acetic acid to succinic acid taking place during alcoholic fermentation. $\text{CH}_3\text{COOH} + \text{CH}_3\text{CHO} \xrightarrow[\text{2}]{+\text{O}} \text{COOH-CH}_2\text{-CH}_2\text{COOH} + \text{H}_2\text{O}$. Concurrently the formation of iso-amyl alcohol, butyl alcohol, citric acid, carbonic acids and stearines with acetate as an intermediary product were observed. Only the 2,3-butylene glycol and ethyl alcohol remained unmarked. The previous assumption by Ehrlich that yeast cells utilise amino acids for the formation of fusel oils during fermentation had thus lost some of its importance. These workers suggested the following synthesis:-



From acetone and acetaldehyde, b-methyl crotonaldehyde is synthesised by the yeasts. This substance is then reduced to amyl alcohol.

Ehrlich¹²⁾, Neuberger and Hildesheimer¹³⁾ showed that a condition for the formation of higher alcohols from amino acids by the yeasts, is a simultaneous conversion of large

amounts.....

amounts of sugar to ethyl alcohol. During the cell-free fermentation of sugar, according to Ehrlich, Buchner and Meisenheimer¹⁴⁾ no fusel oils are formed. Although the mechanism of the transformation of carbohydrates to ethyl alcohol has been intensively studied, its relation to the Ehrlich mechanism and other chemical processes has not been explained in detail.

The theory on fusel oil formation from amino acids as put forward by Ehrlich together with the observation that cell-free fermentation does not yield any fusel oil, prompted Castor and Guyon¹⁵⁾ in the course of a fermentation study on grape juice and with the addition of pure culture yeasts, to determine at regulated intervals the amount of alcohol, fusel oil, valine, leucine and isoleucine in the substrate. At the same time, yeast counts were made. Thirty-seven hours after commencement of fermentation, when the concentration of the three amino acids had reached a minimum value and the yeast count showed its maximum, only 34% of the theoretical amount of fusel oil had been reached. After 168 hours, at which stage the ethyl alcohol concentration had reached its maximum, 255% of the theoretical fusel oil value had been reached. The explanation of this phenomenon, when adopting the Ehrlich viewpoint, is that the keto-acids which are initially formed through deamination, i.e. surrender to their NH_2 groups as NH_3 to the yeasts, can only slowly be decarboxylated and subsequently reduced to alcohols, according to Neubauer and Fromherz¹⁶⁾. The formation of both alcohol and fusel oil are in continuous competition as both reactions are governed by the same initial process. The enzymes are carboxylase and the hydrogen carrying alcohol dehydrogenase, co-enzyme I in the zymase system.

The finding that appreciably more than the theoretically expected amount of fusel oil is formed, is explained by Castor and Vosti¹⁷⁾ as being due to a supplementary delivery of amino acids from the decaying (autolyzing) yeast cells.

At this stage, such cells have stopped their multiplication. Castor and Guyon¹⁸⁾ contend that the Ehrlich mechanism still holds good.

The biological destruction of the acids is also of major importance in this respect. It has long been recognised that the malic-lactic acid formation can be attributed to a certain group of bacteria. Müller-Thurgau and Osterwalder¹⁹⁾ isolated bacterium *gracile* from wine. For the growth of these bacteria, specific nitrogenous substances are necessary.

Archinard²⁰⁾ found that the tartaric acid destroying bacteria do not influence the NH_3 content, whilst bacterium gracile reflects itself in an appreciable increase in the NH_3 content.

A balance of the different nitrogenous compounds in musts and wines was worked out by Hennig and Ohske^{21 & 22)}. The nitrogen balance offered an insight in to the participation of the individual nitrogen containing compounds (or groups of compounds) in the total nitrogen content. Further, Hennig and Flintje²³⁾ investigated changes in the amino acids during and after vinous fermentation. It was found by these authors that up to five days after commencement of fermentation, the amino acids decline as they are required by the yeasts themselves. After five days, a slow increase of amino acids is observed.

The yeasts in a natural must are stimulated in their normal development through the addition of NH_4 salts. According to Prillinger and Saller²⁴⁾ they are unable to utilise NH_4 -nitrogen alone.

Schanderl²⁵⁾ contends that it is a general characteristic of yeast cells to form proteins from simple ammonium salts. Nitrates are of no use as a source of nitrogen. The extent to which an amino acid can be assimilated is governed by the position of the NH_2 group in its molecule. Preference is shown towards amino acids which have their NH_2 group substituted at the β -carbon atom, whilst those having their NH_2 group attached to the α -carbon atom are not assimilated. The length of the carbon chain also influences the extent to which an amino acid might be assimilated, those showing a low molecular weight, being more easily assimilated. With aromatic amino acids, only the nitrogen-containing side chain is assimilated. The yeasts apparently have the ability to affect intramolecular re-arrangement of certain substances so as to render them more easily accessible. Certain amino acids tend to present characteristics bearing close resemblance to those showed by certain "growth substances". Nielsen²⁶⁾ found that the speed with which a particular yeast precipitates to form a sediment can be influenced by its nitrogen supply. Yeasts nourished with amino acids precipitate more rapidly than yeasts nourished with inorganic nitrogenous compounds.

According to Davis²⁷⁾ the amino acids in musts have their origin in the carbohydrates chiefly in glucose, which in this respect seems to play the major role. He contends that

the difference drawn between biosynthetic (catabolic) and degradative (anabolic) processes, remain purely artificial. Important processes do not only supply energy but also the compounds essential for further biosynthesis.

The tricarboxylic acid cycle is the source of glutamic and aspartic acids and their derivatives. Oxidised glucose via phosphogluconate yields ribose and heptose, which in all probability fulfil a very important role in the biosynthesis of aromatic substances. Pyruvic acid, an immediate precursor of alanine, valine and leucine, by way of the acetyl-coenzyme A path, is also of primary importance in the synthesis of many other compounds.

The most important step in the transformation of ammonia to an α -amino group is most probably the action of the enzyme glutaminedihydrogenase, which forms glutamic acid from α -ketoglutaric acid and ammonia. This enzyme has recently been crystallised. Diphosphopyridinenucleotide (DPN) is the hydrogen donor in many organisms, whilst (TPN) triphosphopyridinenucleotide is the hydrogen donor in others. The NH_2 group is transferred by the glutamic pyruvic acid transaminase to many other keto-acids. All natural amino acids are apparently able to participate in this most important reaction.

The enzyme aspartase can, according to Meister²⁸⁾ initiate the formation of α -amino groups through the transformation of aspartic acid derivatives to fumaric acid derivatives and ammonia. Aspartase also plays an important role in the assimilation of molecular nitrogen and hydroxylamine may be synthesised in this way.

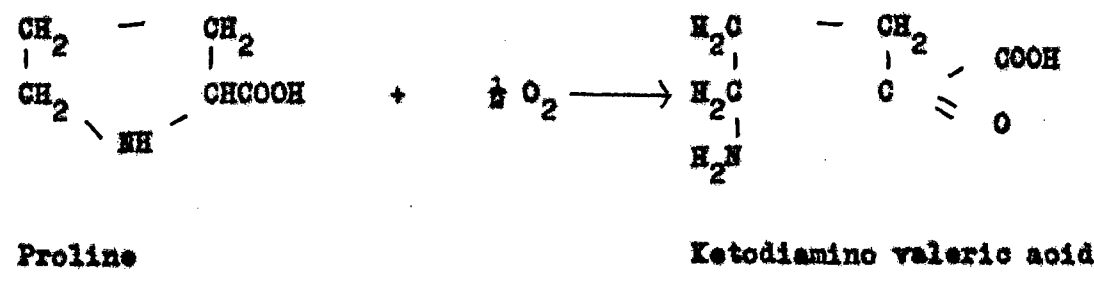
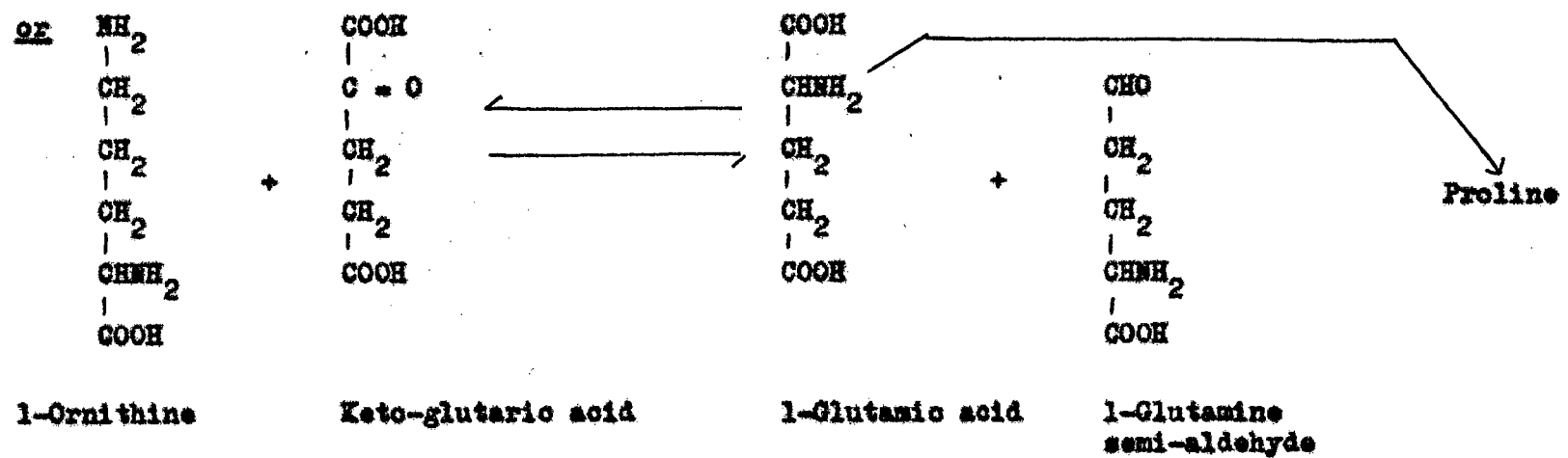
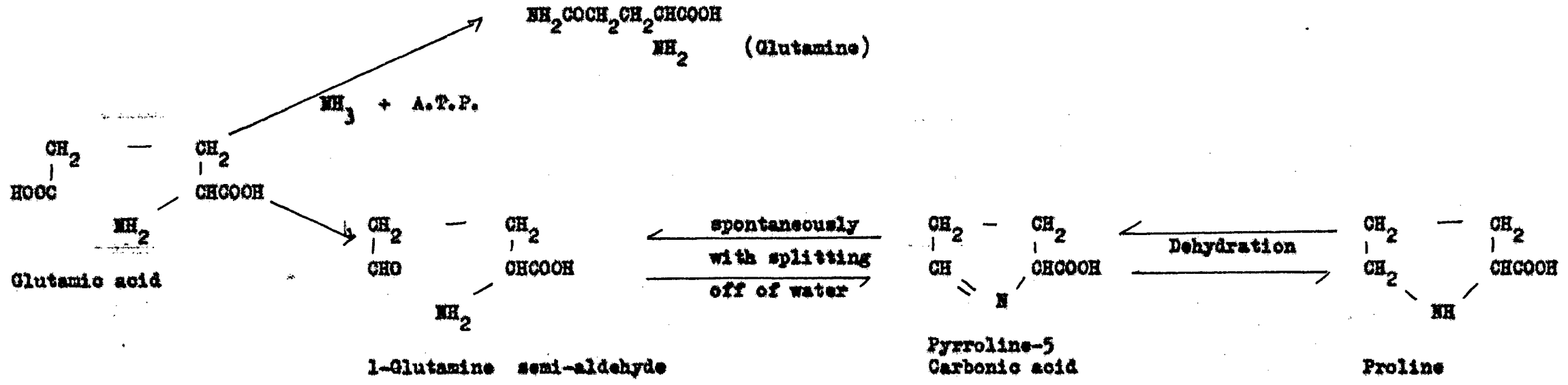
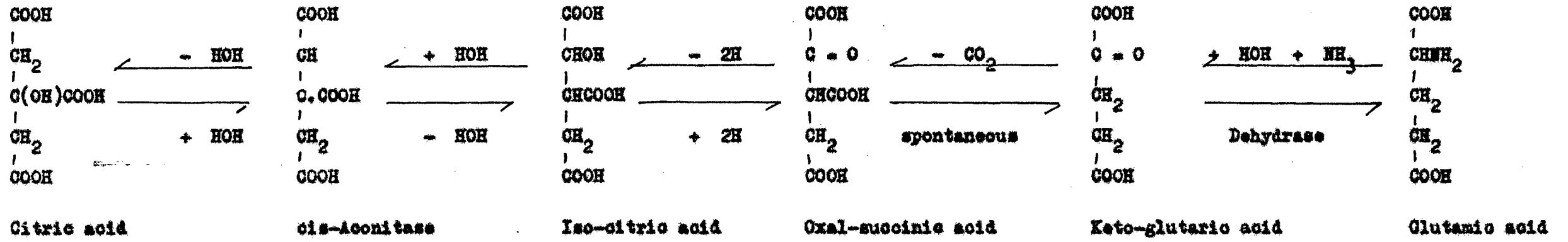
The normal biosynthetic reactions in micro-organisms are the transformation of the corresponding α -keto acids to glutamic and aspartic acids, alanine, phenylalanine, isoleucine, valine and probably tyrosine and leucine. For these amino acid formations, the transaminase always represents the final step in the reaction chain. For the other amino acids, i.e. tryptophane, histidine, lysine, arginine, threonine, methionine and cystine, a transaminase of the corresponding α -keto acid is not compulsory.

Close relationship exists between the citric acid cycle and the amino acid protein metabolism. The intermediary products of this cycle, i.e. α -ketoglutaric, oxal-

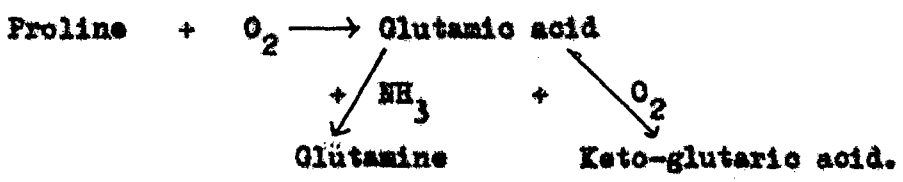
acetic and pyruvic acids can be converted into their corresponding amino acids by specific enzymes.

The α -ketoglutaric acid is transformed by a PN-transhydrogenase (glutamic acid dehydrogenase) to glutamic acid. Furthermore, several transaminases are known which, through the transfer of amino groups by different donors onto α -ketoglutaric acid, facilitate the formation of glutamic acid. Through transamination oxal-acetic and pyruvic acids are transformed into aspartic acid and alanine, respectively. In certain micro-organisms fumaric acid may be of importance as an intermediary product of similar function in the said cycle. Certain enzymes are also known (i.e. the aspartases or aspartase ammonia-lyases) which, through a direct rearrangement of ammonia, catalyses the formation of aspartic acid from fumaric acid.

In studies on yeasts, the existence could in many instances be proved of an enzyme system which catalyses the reactions in the citric acid cycle. Nevertheless, many findings stress the fact that in organisms living under aerobic circumstances, additional mechanisms can also participate in the oxidative degradation of the carbohydrates.

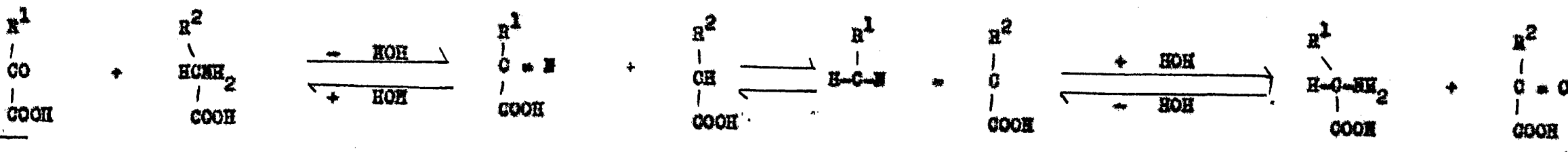


The D (+) Proline is transformed by the D-amino acid oxydase, keto-valeric acid being formed. Thus the same keto-acid is formed as is formed by the degradation of D-ornithine.

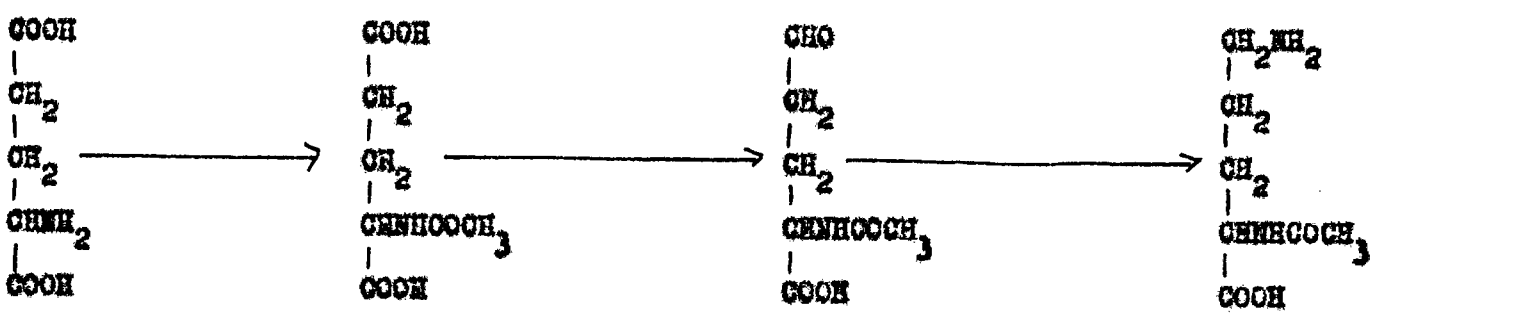


The change of Proline to Glutamic acid corresponds with the observation that Proline can be changed into glucose. 1-Proline possesses no primary amino groups and occurs very widely in vegetable prolamines. It differs from all

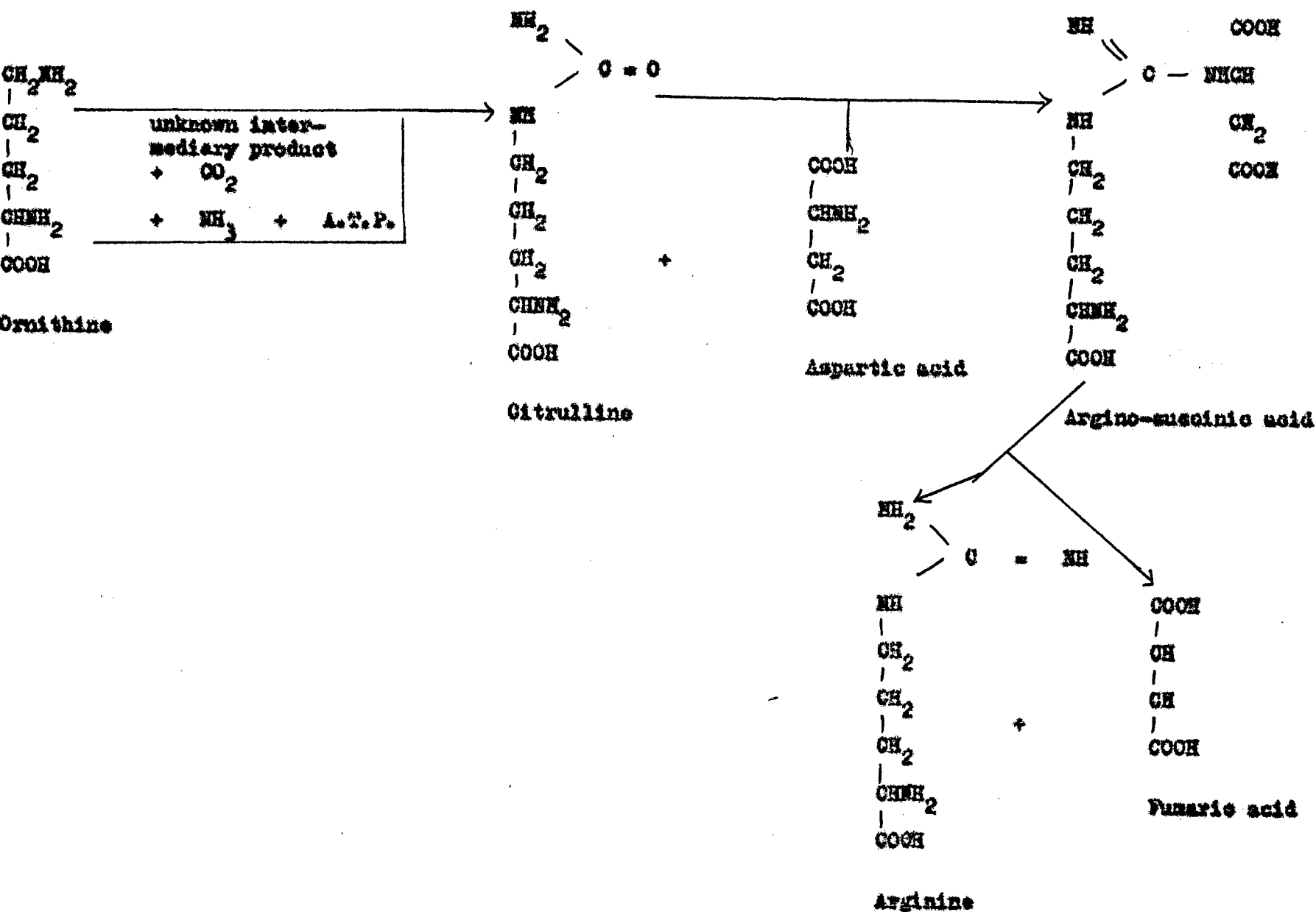
Transamination:



Glutamic acid \longrightarrow Ornithine \longrightarrow Citrulline \longrightarrow Arginine



Glutamic acid N-acetyl glutamic acid N-acetyl glutamic acid semi-aldehyde N-acetyl ornithine



THE AMINO ACIDS IN WINE AND RELATED LIQUIDS.

The first investigations into the amino acids of wines, using paper chromatography, was carried out by the Russians, Ssissakjan and Bessinger²⁹⁾. They identified glutamic acid, alanine, valine and proline. In lesser concentrations they also ascertained aspartic acid, serine, threonine, whilst they remained undecided as to the presence of phenylalanine.

Valaize and Dupont³⁰⁾ established the presence of glycine, alanine, proline, tyrosine, valine, leucine or isoleucine.

The two Swiss workers, Lüthi and Vetsch³¹⁾ agreed with the Russian and French workers and also found a-aminobutyric acid, histidine and arginine to be present. During the course of their investigation on the bacteriological degradation of the acids, they found several bacterial cultures which responded strongly to the addition of specific amino acids. It is a well-established fact that the biological degradation of the acids is subject to seasonal variations, a fact which could possibly be explained by the presence of a particular amino acid grouping. In any case, for the commencement of the acid degradation, a certain degree of autolysis with the resulting formation of amino acids, seems to be a prerequisite. Thus it should be possible to establish differences both in the amino acid level and grouping prior to and after biological degradation of the acids in wine.

Hennig and Flintje³²⁾ and Hennig³³⁾ used paper-chromatographic separation of the amino acids in an endeavour to combine the amino acids with the different phases of development in wine.

Dimotakis³⁴⁾ found 17 different amino acids in Greek vintages from 1947 to 1954, but only from 5-12 amino acids in a particular wine. The wines examined all showed the presence of alanine, aspartic acid, glutamic acid and a high concentration of proline. In addition glycine, leucine, serine, valine, oxy-proline, methionine, norvaline, threonine, arginine, lysine, a-aminobutyric acid, tryptophane, histidine and cysteine were found.

In the course of this investigation up to twenty

different....

different amino acids were found in different wines, but the presence of oxy-proline, norvaline, tryptophane and cysteine could never be established.

d'Almeida³⁵⁾ found fourteen different amino acids in his paperchromotographic investigations on port wine.

Castor³⁶⁾ estimated the quantities of different amino acids in wine by microbiological (lactobacillus) means. In this way he quantitatively determined the amounts of fourteen different amino acids in seven different grape musts. Glutamic acid and arginine generally figured much more prominently than the remaining twelve. Arginine, tyrosine and glutamic acid showed the greatest variation of concentration within the seven different grape varieties examined, while methionine, lysine and leucine showed the least. He also found tryptophane.

Castor and Archer³⁷⁾ determined only those amino acids present in a Colombard must in concentrations higher than 10 mg / 100 ml, before and after fermentation.

	Must mg / 100 ml.	Young Wine mg / 100 ml.
Proline	349	345
Arginine	103	10
Serine	47.9	35.5
Glutamic acid	26.5	2.0
Threonine	21.4	5.1

Prior to fermentation almost three-quarters of the total amino acids (seventeen all told) consisted of proline. This amino acid did not undergo any considerable change in concentration due to fermentation, whilst the serine and threonine diminished by 26% and 76% respectively during the fermentation.

Tarantola³⁸⁾ also applied chromatographic separation for the identification of amino acids in wine. The amino-nitrogen content of the wines examined varied between 9 - 62mg/l. Particularly poor in amino-nitrogen were sweet wines which had been repeatedly filtered, as well as wines resulting from musts immediately separated from their husks. The following amino acids were found:- aspartic acid, glutamic acid, glycine, serine, alanine, proline, leucine or isoleucine, valine,

a-aminobutyric acid, threonine, lysine, arginine, histamine, histidin, tyrosine, cystine, ornithine and asparagine. Of these amino acids other researchers had, up to the time of this author's work, failed to find ornithine, histamine, cystine and asparagine in wine. In all examined wines proline, glycine, serine, alanine, lysine and aspartic acid were found. Histamine, ornithine and tyrosine were only present in a single sample of wine. Proline was the amino acid present in the greatest concentration. Wine left in contact with its lees for a period of one month longer than the control showed an increase in amino nitrogen amounting to 18 mg/l. This increase was chiefly due to increases in the amounts present of the following three amino acids, i.e. valine, leucine and tyrosine. A delay in the time of racking of a wine does not only quantitatively, but also qualitatively influence the amino acid content of a wine. This observation is of both academic and practical interest when the fact is borne in mind that with the occurrence of the malic acid bacteria in wine, certain indispensable amino acids play a decisive role in the malic-lactic acid degradation.

Joslyn and Stepka³⁹⁾ determined the amino acids in apples, apricots, pears and plums. They extracted the fruit with 80% alcohol and afterwards found tyrosine and 3,4-dioxyphenylalanine (Dopa) in the abovementioned fruits. In the course of this study the occurrence of Dopa in grape musts or wines could not be proved although the presence of tyrosine in wine is an established fact.

Koch, et al⁴⁰⁻⁴⁵⁾ investigated the chemical composition of turbidities formed in different flash pasteurised wines and their relationship to the so-called protein turbidities of wines. They contend that the primary aim of modern cellar technology is to render "protein stable" bottled wines. By the application of the generally advocated procedure for rendering wines free from protein turbidities, i.e. flash-heating to 68 - 75°C for two minutes and cooling to -4°C, turbidities easily separated by filtration and of differing chemical composition, are formed.

With the aid of ammonium sulphate they precipitated two different proteins from wine. These two compounds could be separated by paper electrophoresis. The two protein fractions are of widely different stability, fraction I showing a

degree of instability appreciably higher than fraction II. Thus the contention exists that fraction I is primarily responsible for the protein turbidities of wine.

When wine is treated with Geisenheimer Clay (Bentonite) in order to render it protein stable, both protein fractions have to be completely removed. They contend that wine subjected to this high and low temperature treatment have more body than the corresponding bentonite-treated wine.

They hydrolysed the nitrogenous substances which were precipitated according to the Voit method (native protein) with 25% hydrochloric acid and zinc chloride and found the following seventeen amino acids:- alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tyrosine and valine. The protein fraction which showed the greater instability towards heat yielded (after hydrolysis) the abovementioned seventeen amino acids plus cystine. Furthermore, the tartrate-free dry turbid matter showed a tannin content of 3.7% and after hydrolysis a reducing substance value amounting to 12 - 14%.

Koch, et al came to the conclusion that the flash pasteurisation of musts has no influence whatsoever on the subsequent fermentation. The fermentation changes the amino acid composition of the musts as well as causing a partial denaturation (precipitation in alcohol) of the proteins, without in any way changing the numerical amino acid composition of the must protein, i.e. only a portion of the protein is removed, the remaining portion after hydrolysis yielding the same chromatographic picture.

The fact that red wines are free from protein turbidities is explained by the absence of soluble protein in red wine. The chief cause for the protein turbidities of white wines is to be found in this soluble protein fraction.

Lüthi⁴⁶⁾ showed that by rendering musts completely brilliant prior to fermentation from 40 - 60% of the total nitrogen is removed. By such action, albuminous nitrogen is almost completely removed with a subsequent retardation of the general fermentation processes.

Saller⁴⁷⁾ examined the possibilities of controlling the fermentation of apple musts through the removal, or partial

removal, of nitrogenous substances.

Janke and Klemen⁴⁸⁾ showed that the treatment of musts with Lewatit 100 -ion-exchanger renders them unfermentable. This can also be ascribed to the removal of the nitrogen sources.

Rentschler et al⁴⁹⁾ found that especially in wines showing a small amount of still unfermented sugar, the amino acids may seriously harass the reducing sugar determination.

Bourgeois⁵⁰⁾ determined the amino acids in five different vinegars with the aid of paper chromatography and biological methods. It was shown that vinegars produced by fermentation have certain amino acids, whilst those prepared with vinegar essence do not show any amino acids. Thus these two types of vinegar may easily be identified.

Peltonen et al⁵¹⁾ found that the two-dimensional chromatograms of the same wine from different vintages show clearly discernable differences. The chromatograms from different wines show marked differences.

Prillinger⁵²⁾ contends that the number of amino acids generally occurring in wine is comparatively high and usually ranges in the vicinity of twenty. He proves this by showing that during yeast autolysis, with the exception of proline, aminobutyric acid and methionine, fourteen other amino acids are formed. According to his findings the decrease of total nitrogen during normal fermentation amounts to 364 mg/l, which corresponds to an amino acid content of 2280 mg/l.

TABLE I

AMINO ACIDS AS FOUND BY DIFFERENT AUTHORS.

	<u>Amino Acids found in Must and Wine.</u>								
	Ssissakjan, ²⁹⁾ Bessinger	Valaize, ³⁰⁾ Dupont	Lüthi, ³¹⁾ Vetsch	Hennig, ²³⁾ Flintje	d'Almeida ³⁵⁾	Castor ³⁶⁾	Tarantola ³⁸⁾	Koch ⁴⁰⁻⁴⁵⁾ (in hydro- lysed protein)	Own experiments
Glycine		+	+	+	+	+	+	+	+
Alanine	+	+	+	+	+		+	+	+
Serine	+		+	+	+		+	+	+
Cystine					+	+	+		+
Aminobutyric acid			+	+			+	+	+
Threonine	+		+	+	+		+	+	+
Valine	+	+	+	+	+	+	+	+	+
Methionine				+	+	+		+	+
Leucine		+	+	+	+	+	+	+	+
Iso-leucine		?	+	+	+	+	+	+	+
Phenylalanine	?		+	+	+	+		+	+
Tyrosine		+	+	+	+	+	+	+	+
Proline	+	+	+	+	+		+	+	+
Tryptophane						+			
Aspartic acid	+		+	+	+	+	+	+	+
Asparagine							+		+
Glutamic acid	+		+	+	+	+	+	+	+
Arginine			+	+	+	+	+	+	+
Lysine				+	+	+	+	+	+
Ornithine					+		+		+
Histidine			+	+	+	+	+	+	+
Histamine							+		

SUMMARY OF THE METHODS OF INVESTIGATION.

Oreskes and Seifert⁵³⁾ used the round chromatogram technique to determine the amino acids in certain protein hydrolysates. They simultaneously developed two round chromatograms, using phenol-isopropyl alcohol : water (70:5:25) as the one and butanol : ethyl alcohol : water (40:10:50) as the other developing solution. The dried papers were halved, the one half bathed in a solution of 0.2% isatine in acetone and 4% ethyl alcohol, and the other half in 0.25% ninhydrin in acetone. Good Rf-values were obtained.

Krisnamurthy and Swaminathan⁵⁴⁾ also applied the round filter technique for the estimation of amino acids in casein hydrolysates. The dried chromatograms were afterwards sprayed with 0.5% ninhydrin in methyl alcohol to which 5% ethyl alcohol had been added. The ninhydrin spots were extracted with 75% ethyl alcohol and colorimetrically estimated at 560 millimicrons. In this way it was possible to recover 95 - 103% of added amino acids.

Lakshihimarayan Rao⁵⁵⁾ chromatographically separated the different amino acids in mixtures containing up to seventeen different amino acids on Whatman No. 1 paper, previously treated with 0.2 M-potassium chloride - hydrochloric acid, dried and developed downwards with phenol water, fifty volumes of the organic phase mixed with seven volumes of the previously mentioned buffer, dried and subsequently developed in the second direction with butanol : ethyl alcohol : water (4:1:1). The dried chromatograms were bathed in 0.4% ninhydrin (95% ethyl alcohol and 4% acetone) and dried for thirty minutes at 60°C for colour development.

Bozidar and Briski⁵⁶⁾ used a modified ninhydrin method. The developed and dried chromatogram was sprayed with 1% ninhydrin and dried for fifteen minutes at 110°C. Subsequently the paper was once more very lightly sprayed with the same reagent, dried at room temperature for five to ten minutes and thereafter dried for five minutes at 80°C in a chamber saturated with water : ethyl alcohol vapour, containing traces of collidine and lutidine. The amino acid stains are cut out and extracted with ethyl alcohol : acetone : water (1:1:1) and colorimetrically determined with a Pulfrich photometer at 570 millimicrons (proline at 440 millimicrons).

By comparing against a standard extinction graph, individual amino acids could be determined to within an error margin of 6%.

Lüthi and Vetsch^{57 & 58)} in their comprehensive work on the amino acids in wines used a one-dimensional method as first suggested by McFarren⁵⁹⁾. They simultaneously developed chromatograms in different solutions, i.e. phenol, m-cresol, benzylbutyl alcohol. The amino acid mixtures are spotted on buffered (pH 12.0 and 8.4) Whatman No. 1 paper.

Koch⁶⁰⁾ used the same technique but with Schleicher and Schüll 2043 paper.

Seelkopf and Schuster⁶¹⁾ after a detailed study of the different methods of hydrolysis contended that hydrolysis in acid medium is preferable to hydrolysis in alkaline medium. During acid hydrolysis loss of amino acids is considerably less than otherwise. Also when applying acid hydrolysis, catalysts, like the mercury salts for instance, can be employed, enabling the time of hydrolysis to be considerably shortened. They also found that in natural products where proteins are always accompanied by carbohydrates, prior separation of the said substances becomes imperative. Heating for five hours with 5N formic acid in a hermetically-sealed glass tube transforms the starches into easily soluble compounds, while the proteins are only transformed to peptides, thus rendering them unable to react with the sugars. The peptides and amino acids are precipitated. For the qualitative estimation of the amino acids they used a two-dimensional method with phenol (pH 12) as the first and butanol : acetic acid : water as the second developing solution. For quantitative determination they applied the method of McFarren. The phenol is removed very carefully in a drying cupboard at 40 - 60°C under vacuum. The dried chromatogram is bathed in 5% ninhydrin in butyl alcohol (+ 5% glacial acetic acid) and again dried. The spots are cut out and extracted on a warm waterbath with 2 ml. of a 5% ninhydrin solution in butanol in ethyl glycolmonomethyl ether for colour development. To this solution is then added 5% ninhydrin solution in n-propyl alcohol, total volume amounting to 10 ml. The extinction is determined in a Pulfrich photometer at 670 millimicrons. Proline is measured at 440 millimicrons.

Bayer, Reuther and Born⁶²⁾ suggested that the amino acid esters could rapidly be separated by means of the gas phase chromatography as first suggested by James and Martin.

The amino acid mixtures are suspended in methyl alcohol and hydrochloric acid (gas) is induced. The amino acid methyl-esterhydrochlorides formed are neutralised and the free esters extracted with di-ethyl ether and subjected to a separation by gas chromatography.

Wunderley⁶³⁾ in his comprehensive work on the paper electrophoresis, i.e. the principle of separation of different substances in an electrical field based on their different rates of movement, mentions the fact that this principle was already adopted by Field and Teague⁶⁴⁾ as far back as 1907. The latter separated the diphtheria toxin and anti-toxin on an agar gel. The first experiments were all carried out on different gels. Later different clays started finding application. Afterwards the electrolytes were stabilised by using inert substances like powdered glasswool, agar, gelatine, cotton, asbestos, silk and artificial rosins, etc. In 1937 König⁶⁵⁾ used paper as carrier medium. Thus the paper electrophoresis is actually a few years older than the paper chromatography introduced in 1944 by Consden, Gordon and Martin⁶⁶⁾. Work carried out since then clearly showed a desire to combine these two techniques. During the modern development of paper electrophoresis the separation of the proteins and amino acids acted as an important stimulant. Wieland and Fischer^{67 & 68)} and Wieland and Pfleiderer⁶⁹⁾ were the first to separate the amino acids by high tension electrophoresis. This technique was also to some extent adopted in this investigation.

Instead of identifying different substances by their rate of movement Gross⁷⁰⁾ used solutions in different pH-ranges (2.0 : 2.9 : 9.2) to separate different mixtures. He contended that the separation is not governed by mobility alone, but also by the particular qualities of the original spot, i.e. whether it be round or elongated, big or small.

The higher the concentration of the individual substances to be separated, the more pronounced the inclination towards blotching.

In the first separation of an amino acid mixture Blass⁷¹⁾ used a solution of butyl alcohol : acetic acid : water as developing agent. After drying the chromatogram was electrophoretically developed in the second direction at 90° to the first, using Veronal Buffer (pH8.6).

PREPARATION OF THE SAMPLES.

During the course of this study a method was evolved whereby the preparation of samples became limited to an EK-filtration.

Concentration of the sample, to say one-quarter of its original volume, was unnecessary because the strongly represented amino acids like proline and arginine reached such concentrations that they at times completely swamped those present in very much smaller quantities. With solutions high in sugar content concentration only stimulates unnecessary blotching. When heating a wine or must the additional hazard of hydrolysis exists.

The separation of the amino acids by means of a basic ion-exchanger and subsequent desalting, according to Decker, in an apparatus for demineralisation as first developed by Consden, Gordon and Martin, became unnecessary by the method described here. A concentration under vacuum or a concentration as proposed by Lüthi, Koch and Prillinger was also superfluous.

Stein and Moore⁷³⁾ found that with the electrolytic demineralisation according to Consden, Gordon and Martin, the arginine concentration was greatly diminished, ornithine being formed. The loss amongst the remainder of the amino acids was found to be negligible.

Waldron-Edward⁷⁴⁾ observed that with paper electrophoresis when very strongly acid or basic solutions are employed, disturbances due to the presence of small quantities of inorganic salts could be disregarded.

Joslyn and Stepka⁷⁵⁾ found that chromatographically pure solutions of single amino acids, i.e. solutions yielding only a single spot after two-dimensional chromatography prior to hydrolysis, often present multiple spots after hydrolysis.

Klinger⁷⁶⁾ demonstrated a positive ninhydrine reaction on paper chromatograms containing reducing substances, i.e. ascorbic acid or reducing sugars in the presence of traces of ammonia.

Hackmann and Lazarus⁷⁷⁾ found that high concentrations of glutamic acid in a mixture often results in unreliable quantitative values for the remaining amino acids.

SPRAY REAGENTS.

Curzon and Giltrow⁷⁸⁾ found that spraying ornithine with 2% vanilline in n-propyl alcohol results in a strong yellow fluorescence in U.V. light. When treated with vanilline, dried and subsequently sprayed with 1% sodium hydroxide in alcohol, ornithine is stained a dark pink.

According to Blazek⁷⁹⁾ ornithine forms on spraying with α -naphthylamine-diazonium-hydrochloric acid an orange-yellow stain, which presents a dark red coloration under U.V. light.

The basic amino acids arginine, histidine, lysine and ornithine can be identified by spraying with Folin's Reagent, according to Merck⁸⁰⁾ and Kramer⁸¹⁾ and with the sodium salt of 1,2-naphthaquinone-4-sulphonic acid and diazotised sulphanilic acid according to Kramer.

Barollier^{82 & 83)} added small quantities of cadmium chloride to the ninhydrin reagent thus transforming the dyes into metal complex compounds. By the addition of zinc acetate to isatine he also attained a pronounced coloration. Such substances have the advantage that they possess very slight solubility in water and the reagent excess can thus easily be removed. Afterwards the treated chromatograms are lightly impregnated with a diluted varnish without rendering the paper transparent. By this expedient the brilliance and durability of the colour tones are enhanced.

According to Dent⁸⁴⁾ the amino acids are coloured in the following manner by the ninhydrin treatment:-

Purple: Alanine, α -aminobutyric acid, arginine, glutamic acid, leucine, isoleucine, methionine, ornithine, phenylalanine, serine, threonine, tryptophane and valine.

Bluish-purple: Aspartic acid.

Greenish-purple: Phenylalanine and tyrosine.

Dark-blue: Lysine.

Orange-brown: Asparagine.

Lemon-yellow: Proline.

The addition of small quantities of glacial acetic acid to the ninhydrin somewhat dampens the colour contrasts, but in return renders them more apparent.

MOBILE PHASES.

Holland and Nayler⁸⁵⁾ estimated the Rf-values (multiplied by 100) of all the amino acids which generally appear in biological material. They used fifty-five different developing solutions. Treatment of the chromatograms with cyclohexylamine or dicyclo-amine prior to spraying with ninhydrin, resulted in a surprising colour range for the amino acids. These reactions for the identification were partly employed in the course of this work in order to establish a topographical chart for the wine amino acids.

THE QUALITATIVE DETERMINATION OF THE AMINO ACIDS
IN FRESH AND FERMENTING GRAPE MUSTS.

The following method has the advantage that no disturbances due to the presence of carbohydrates, proteins and mineral substances are to be feared.

It is a two-dimensional method. The separation in the first direction is undertaken by electrophoresis. The harassing components remain in the vicinity of the original spot. The second run is undertaken applying the ordinary separation by paper chromatography. By this process the individual amino acids which have not been completely separated after the first run are now separated.

When a must is to be examined it is previously sterilised by filtration.

From a must sample containing a total amino acid content of from 2 - 4 gm/l, an aliquot corresponding to approximately 0.05 mg. is spotted on the paper. The paper used is Schleicher and Schüll, 2043b Mgl., dimensions 30 x 40 cm. The starting point is situated 6 cm. from the longer side (40 cm.) of the paper and 10 cm. from the shorter (30 cm.) side.

Subsequently the pherogram is developed electrophoretically according to Wieland and Pfleiderer⁸⁶⁾. As buffer solution, formic acid : glacial acetic acid : water (pH 1.9) was used. The potential difference amounted to between 1600-1800 volt at 20 - 30 milliamps. The temperature is maintained at -2°C. Subsequently the paper is dried at 90°C for ten minutes.

The dried chromatogram is turned at right angles to the direction of the first separation and developed upwards for twelve hours with phenol : water (7:3) as the mobile phase at a

temperature.....

temperature of $25 \pm 1^{\circ}\text{C}$. Thereafter the chromatogram is dried for fifteen minutes at 100°C .

Spray reagent: 0.2% ninhydrin solution in 95 ml. n-butyl alcohol and 5 ml. of 2N acetic acid.

The chromatogram is dried for ten minutes at room temperature and subsequently for ten minutes at 75°C . The optimal colour formation is observed after twenty-four hours.

In the electrophoresis the distance travelled by the different amino acids is referred to that travelled by l-alanine according to a method first adopted by Wieland.

The Rf-values obtained with an artificial must to which amino acids had been added, developed with phenol : water, showed constant values for repeated runs.

In a number of musts up to twenty different amino acids were found which was not at all surprising when a total amino acid content of from 2 - 4 gm/l seems to be the rule.

Musts were also found in which certain amino acids, i.e. glycine, serine, α -aminobutyric acid, threonine, cystine and asparagine were not detectable.

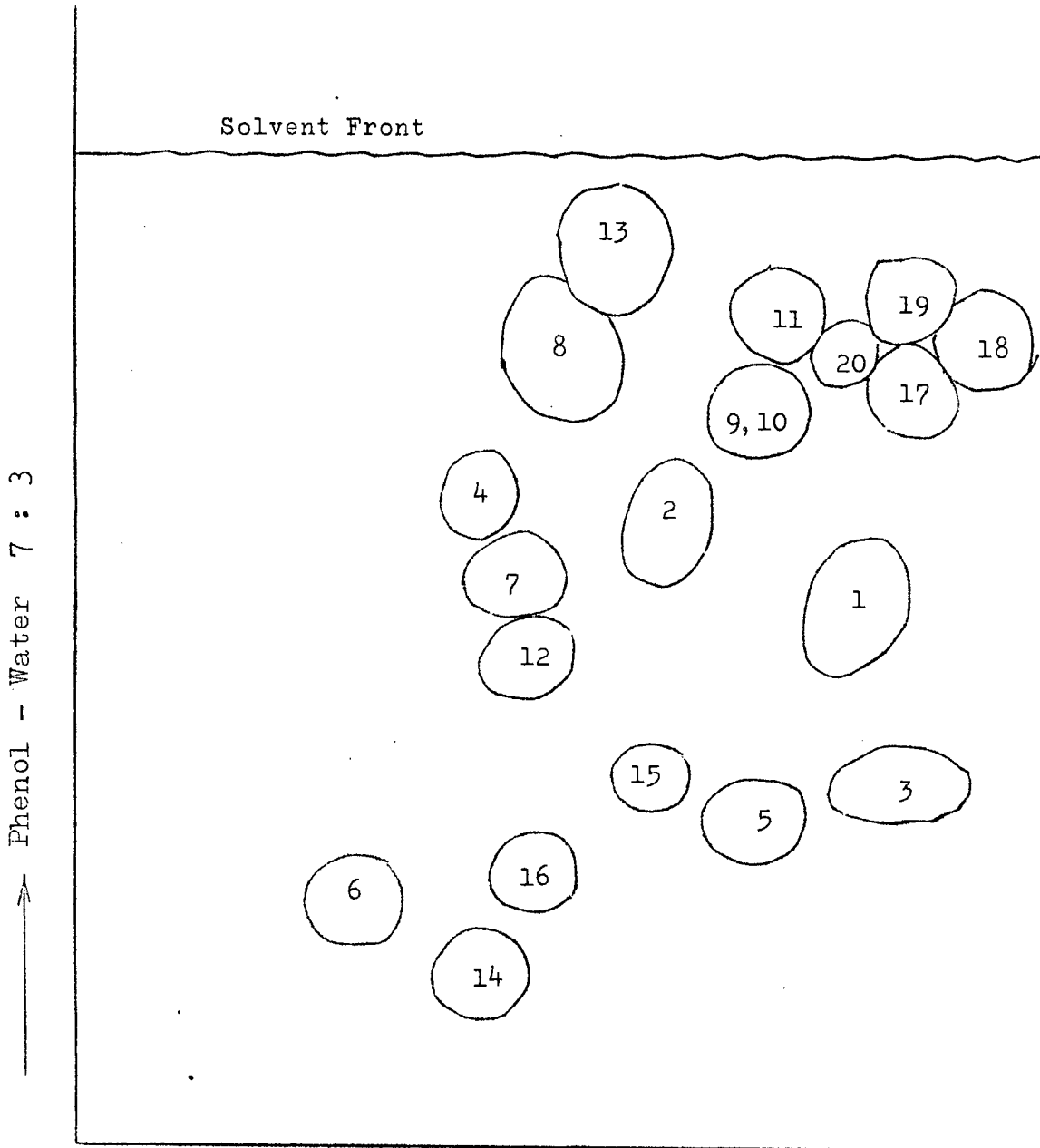
TABLE II. THE R_{Alanine} VALUES OBTAINED BY ELECTROPHORESIS AND R_f -VALUES (PHENOL : WATER) FOR TWENTY DIFFERENT AMINO ACIDS.

	Electrophoresis R_{Alanine}	Chromatography $R_f^{\text{Phenol:Water}}$ 7:3
1. Glycine	1.23	0.53
2. l-Alanine	1.00	0.62
3. Serine	1.27	0.36
4. Cystine	0.83	0.64
5. α -Aminobutyric acid	1.08	0.33
6. Threonine	0.57	0.25
7. Valine	0.87	0.57
8. Methionine	0.88	0.83
9. Leucine	1.10	0.74
10. Isoleucine	1.10	0.74
11. l-Phenylalanine	1.14	0.81
12. Tyrosine	0.85	0.52
13. Proline	0.88	0.90
14. Aspartic acid	0.68	0.18
15. Asparagine	0.98	0.37
16. Glutamic acid	0.79	0.26
17. Arginine	1.31	0.77
18. Lysine	1.44	0.81
19. Ornithine	1.35	0.82
20. l-Histidine	1.24	0.79

Diagram I.....

Diagram I.

Topographical Chart of 20 Different Amino Acids according to Data supplied in TABLE II, page 25.



Electrophoresis: V. 1800 mA 20 Hrs. 2
pH 1.9 Temp. -2°C.

THE QUANTITATIVE DETERMINATION OF THE AMINO ACIDS.

For the quantitative determination of the amino acids from 200 - 300 micrograms of amino acids are spotted on the paper. As it remains difficult to spot more than 0.05 ml, the mentioned quantity is equally divided between one to four chromatograms. For a fermenting must more material is required than for the original must or finished wine. The volume brought onto the paper is maintained at 0.05 ml so as to render the chromatograms for the same sample as nearly identical as possible.

The further procedure is the same as that discussed under the qualitative determination, excepting that after development in the second direction (with phenol:water) the chromatogram is not immediately dried at 100°C. The chromatograms are hung in the drying oven overnight and the following morning dried for fifteen minutes at 40°C and subsequently at 100°C as previously described. Twenty-four hours are allowed to elapse for maximum colour development. For comparison 0.2 ml. must or wine can be stroked onto the paper leaving a stripe 20 - 25 cm. long, 10 cm. from the narrow end of the paper and developed uni-dimensionally by electrophoresis.

After optimal colour formation the spots are cut out and cut into small shreds, placed in a test tube and extracted four times, each time using 2 ml. of a solution of acetone : ethyl alcohol : water (1:1:1). Each separate extraction lasts one hour. To the combined extract is added 0.5 ml. of a 0.2% ninhydrin solution in 95 ml. of n-butyl alcohol and 5 ml. 2N-acetic acid to which has been added 75 mg/l of cadmium chloride. The volume is brought up to 10 ml. with distilled water and the solution shaken.

The extinction is measured in a Pulfrich photometer or a Zeiss Elko III, using 2 cm. cuvettes. The extinction for all amino acids, excepting proline, is ascertained at 570 millimicrons, proline being measured at 440 millimicrons. As a blank for comparison an unstained portion of the chromatogram to be examined is cut out and treated exactly as previously described.

The presence of protein at the starting point can be shown by bathing with amido-black 10B in methyl alcohol, nine volumes being added to one volume of glacial acetic acid. This method was introduced by Grassmann et al⁸⁸⁾. The presence of sugar can be ascertained as indicated by Grüne⁸⁹⁾ by spraying

with.....

with analine phthalate.

During and after fermentation the concentration of many amino acids decline rapidly and some others may not be completely separated by chromatography. Thus the determination of the amino acids is undertaken according to the following grouping:-

1. Proline as proline and converted into alanine.
2. Arginine.
3. Glutamic acid.
4. Leucine and isoleucine.
5. Alanine.

It often becomes necessary to group glutamic acid, leucine, isoleucine and alanine together.

6. Phenylalanine, ornithine, lysine, histidine.
7. Serine.
8. Threonine.

Circumstances may, at times, compel the grouping of the last two.

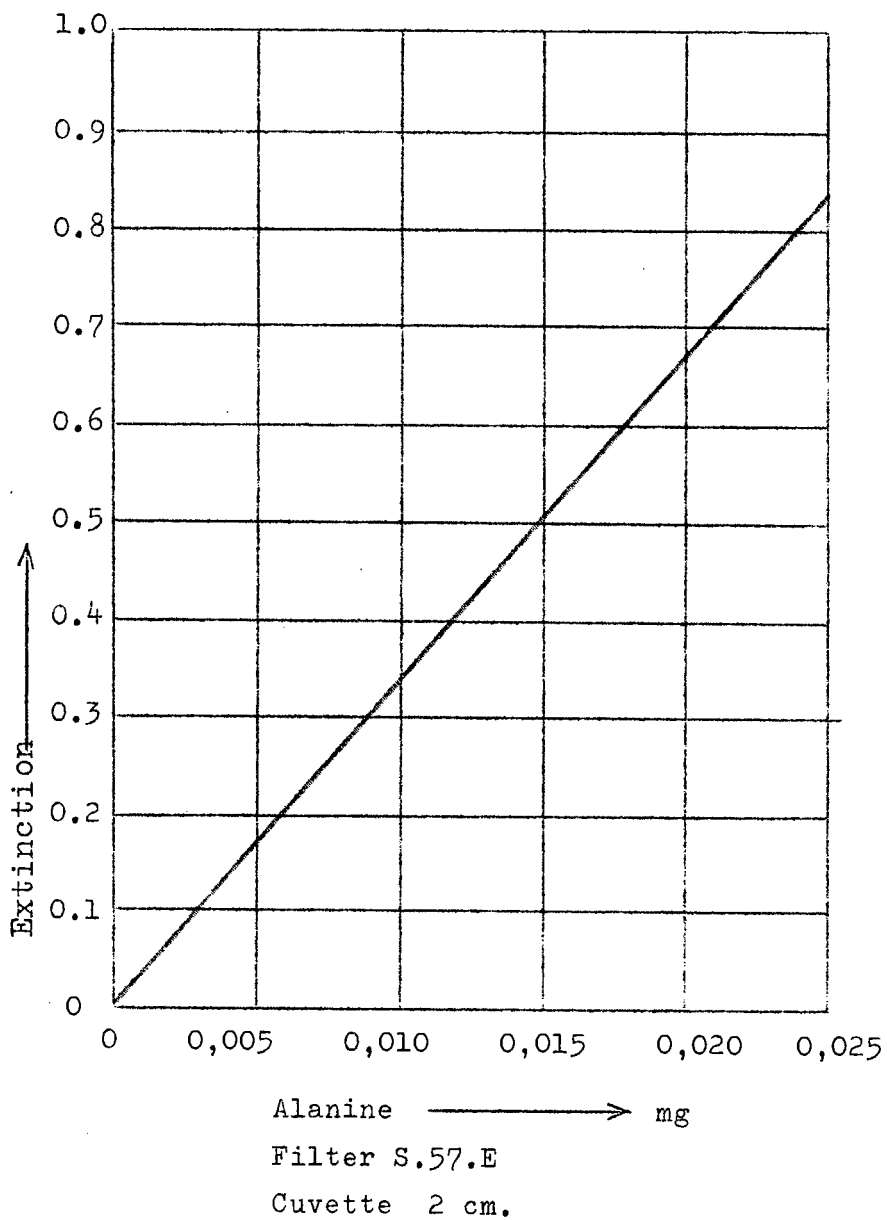
9. Glycine, aminobutyric acid, asparagine, methionine, valine, tyrosine, cystine and aspartic acids.

To arrive at a standard curve the following procedure is adopted. A standard solution containing exactly 1 mg. per ml. of solution (alanine and proline) is prepared and spotted with an Agla micrometer syringe (Burroughs Welcome Co. Ltd., London) in exactly controlled volumes onto Schleicher and Schüll 2043b paper. The spots are dried and sprayed with ninhydrin and further treated exactly as previously described. The extinction was measured in either a Pulfrich photometer or Zeiss Elko III, the values showing good comparison for the two instruments.

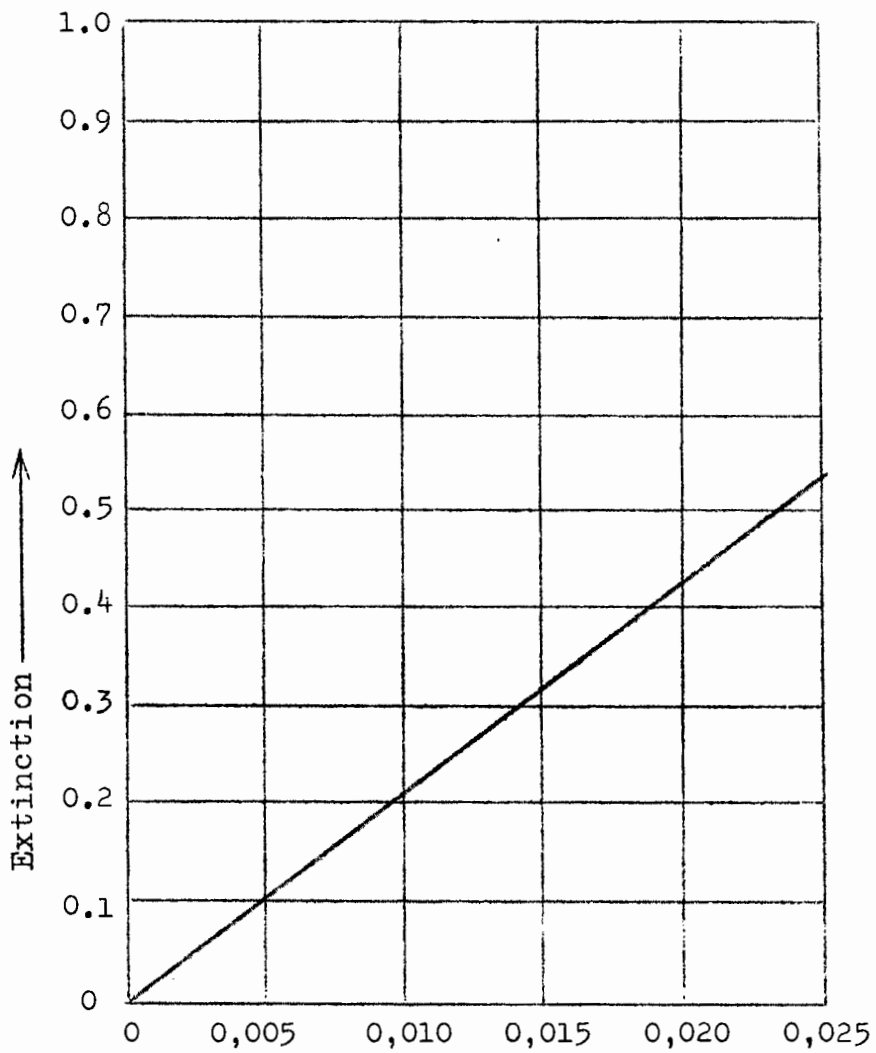
TABLE III. EXTINCTION VALUES FOR ALANINE AND PROLINE IN ZEISS ELKO III PHOTOMETER, 2.0 cm. MEASURING CELLS, ALANINE AT 570 millimicrons AND PROLINE AT 440 millimicrons.

mg. Alanine	Extinction	mg. Proline	Extinction
0.005	0.160	0.005	0.102
0.010	0.332	0.075	0.158
0.015	0.492	0.125	0.262
0.020	0.640	0.150	0.322
0.025	0.805	0.200	0.422

Graph No. 1. Standard Graph for the Determination of Alanine.



Graph No. 2.

Standard Graph
for the Determination of Proline.

Proline → mg

Filter S.42.E

Cuvette 2 cm.

As a check for the procedure 10, 50 and 100 mg. (referred to alanine or proline) of different pure amino acids were dissolved in 100 ml. of water and estimated. The average values for ten determinations are supplied.

TABLE IV. THE RECOVERY OF ADDED AMINO ACIDS IN PURE SOLUTION.

Pure Substance	mg.	Estimated as	Average Value for ten Determinations
Proline	10	Proline	10.6 mg
Proline	50	Proline	48.0 "
Alanine	10	Alanine	9.8 "
Alanine	50	Alanine	51.6 "
Arginine	10	Alanine	10.4 "
Arginine	50	Alanine	47.7 "
Mixture of five amino acids, arginine, glutamic acid, valine, leucine and serine	50	Alanine	54.4 "
	100	Alanine	95.6 "

A wine was examined by the above method and found to have 196 mg/l total amino acids (proline excluded), estimated as alanine. To this wine a mixture containing the five amino acids arginine, glutamic acid, valine, leucine and serine was added and afterwards subjected to an analysis for total amino acids. A satisfactory recovery was registered.

TABLE V. THE RECOVERY OF ADDED AMINO ACIDS IN WINE.

Addition of Amino Acids to Wine mg/l	Theoretical Total Amino Acids in Wine (as Alanine) mg/l	Total Amino Acids Determined in Wine (as Alanine) mg/l
50	246	240
200	396	402

The same procedure was adopted by adding proline (as proline) to a wine showing a proline content of 156 mg/l. The proline was estimated as proline.

TABLE VI. THE RECOVERY OF ADDED PROLINE IN WINE.

Addition of Proline to Wine mg/l	Theoretical Quantities of Proline in Wine mg/l	Proline Determined in Wine mg/l
50	206	200
100	256	265

PRACTICAL INVESTIGATIONS.

The first investigations were carried out with a Geisenheimer Morschberg wine originally showing a sugar content of 60° Oechsle and 12.6 gm/l total acid. To this must sugar solution was added, increasing the volume by 20% and the sugar to 90° Oechsle. With the aid of calcium carbonate the total acids were decreased (neutralised) until they presented a value of 9 gm/l. The must was filled into a large tank and one half was subsequently centrifuged. The naturally turbid and centrifuged musts were filled into 600 litre vats.

Vat No. 1 contained the original must, i.e. in its state of natural turbidity. Vat No. 2 contained the same sample centrifuged. To Vat No. 3 containing the naturally turbid must and Vat No. 4 containing the same must, centrifuged liquid panzym (Firma C.H. Boehringer Sohn, Ingelheim) was added in the proportion of 50 ml/100 litre of must. To Vat No. 5 (originally turbid) and Vat No. 6 (centrifuged) 50 gm. of dry panzym to 100 litres of must were added.

The panzym is held to aid in the destruction of the harassing pectin compounds in grape and berry juices. It is also generally contended that it enhances the flavour.

The grapes of the Geisenheimer Morschberg vineyard were harvested during November, 1956. Immediately following the crushing operation a total analysis of the must as well as a qualitative uni-dimensional electrophoretical estimation of the amino acids was undertaken. On the third day after crushing a gentle fermentation could be observed. The fermentation proper took place in the different samples from the fourth to the seventh day.

During and after fermentation samples were drawn for the determination of total nitrogen, amino acids and for detailed analysis. The yeasts were removed by centrifuging and the various analyses of must or young wine were undertaken with completely clear samples. The samples were withdrawn on the 3rd, 6th, 10th, 13th, 20th and 32nd days after crushing.

TABLE VII

ANALYSIS OF MUSTS IMMEDIATELY AFTER CRUSHING.

1956 Geisenheimer Morschberg.

Original must composition: 60.3° Oechsle. 12.6 gm/l. Total Acidity.
 20% Improvement and Treatment with CaCO₃.
 Composition after improvement and treatment: 90.0° Oechsle. 9.0 gm/l. Total Acidity.

First Day	1. Turbid Original	2. Clear Original	3. Turbid Panzym liquid	4. Clear Panzym liquid	5. Turbid Panzym solid	6. Clear Panzym solid
Specific Gravity	1.0840	1.0840	1.0855	1.0838	1.0845	1.0841
Total Acid gm/l.	9.4	9.2	9.2	9.1	9.5	9.1
Tartaric Acid gm/l.	4.5	4.6	4.4	4.6	4.4	4.6
Malic Acid gm/l.	4.2	4.3	4.3	4.3	4.3	4.3
Lactic Acid gm/l.	0.1	0.1	0.1	0.1	0.1	0.1
Ash gm/l.	3.39	3.18	3.22	3.36	3.25	3.34
Ash Alkalinity (ml. of N. NaOH/liter)	44.0	44.7	42.7	43.0	45.7	43.3
Total Sulphur-dioxide mg/l.	37	25	25	22	23	23
Free Sulphur-dioxide mg/l.	7	4	4	4	4	4
Fixed Sulphur-dioxide mg/l.	30	21	21	18	19	19
Total Nitrogen (N ₂) mg/l.	727	741	759	776	735	776
pH-Value	3.19	3.19	3.19	3.19	3.19	3.19
Volatile Ester mg/l.	60	60	56	56	63	63

TABLE VIII

ANALYSIS OF MUSTS 32 DAYS AFTER CRUSHING.

1956 Geisenheimer Morschberg.

Original must composition: 60.3° Oechsle. 12.6 gm/l. Total Acidity.
 20% Improvement and Treatment with CaCO₃.
 Composition after improvement and treatment: 90.0° Oechsle. 9.0 gm/l. Total Acidity.

32nd Day	1. Turbid Original	2. Clear Original	3. Turbid Panzym liquid	4. Clear Panzym liquid	5. Turbid Panzym solid	6. Clear Panzym solid
Specific Gravity	0.9967	0.9961	0.9953	0.9964	0.9968	0.9954
Alcohol gm/l.	94.3	89.2	91.2	87.8	91.2	90.6
Alcohol Vol. %	12.00	11.30	11.56	11.12	11.56	11.48
Total extract gm/l.	32.8	28.1	27.4	29.2	30.7	26.6
Sugar gm/l.	7.7	4.4	3.1	5.1	7.2	3.0
Sugar-free extract gm/l.	26.1	24.7	25.3	25.1	24.5	24.6
Total acid gm/l.	8.1	8.6	8.8	8.5	8.0	8.5
Tartaric acid gm/l.	2.5	2.8	2.7	2.7	2.5	2.6
Malic acid gm/l.	3.9	4.1	4.0	4.1	3.8	3.9
Lactic acid gm/l.	1.0	1.1	1.3	0.9	1.1	1.7
Volatile acid gm/l.	0.2	0.2	0.2	0.2	0.2	0.2
Ash gm/l.	2.19	2.39	2.30	2.30	2.24	2.32
Ash Alkalinity (ml. of N. NaOH/Liter)	26.6	29.3	28.0	28.0	29.3	29.0
Total Sulphur-dioxide mg/l.	97	88	85	78	100	100
Free Sulphur-dioxide mg/l.	10	9	10	9	9	10
Fixed Sulphur-dioxide mg/l.	87	79	75	69	91	90
Total Nitrogen (N ₂) mg/l.	432	479	414	487	417	530
pH-Value	3.14	3.17	3.14	3.17	3.20	3.21
Volatile ester gm/l.	99	102	99	102	102	112

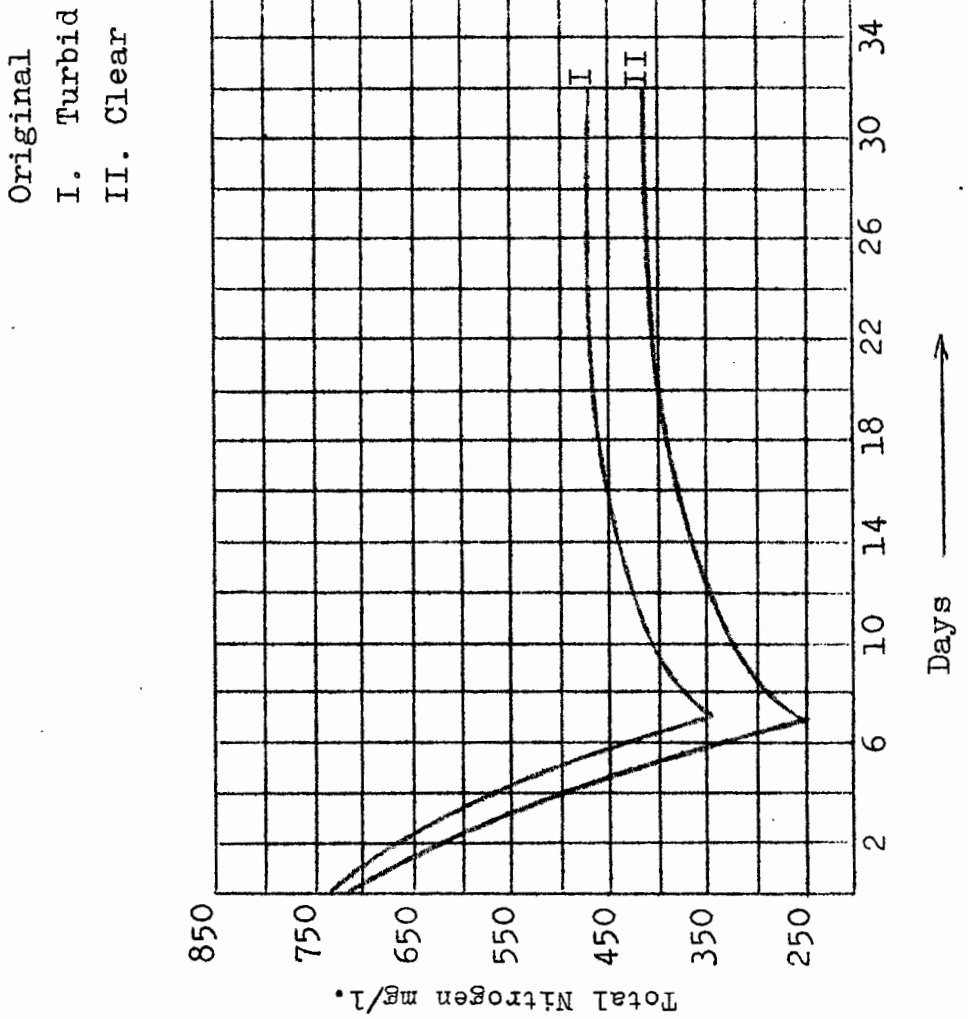
TABLE IX

TOTAL NITROGEN mg/l. IN SAMPLES

Day	1. Original turbid	2. Original centrifuged	3. Panzym liquid turbid	4. Panzym liquid centrifuged	5. Panzym solid turbid	6. Panzym solid centrifuged
0	727	741	759	776	735	776
3	508	642	455	648	461	636
6	327	414	315	420	298	374
10	339	420	368	432	339	394
13	371	438	397	447	377	417
20	406	470	397	490	397	458
32	432	479	414	487	417	530

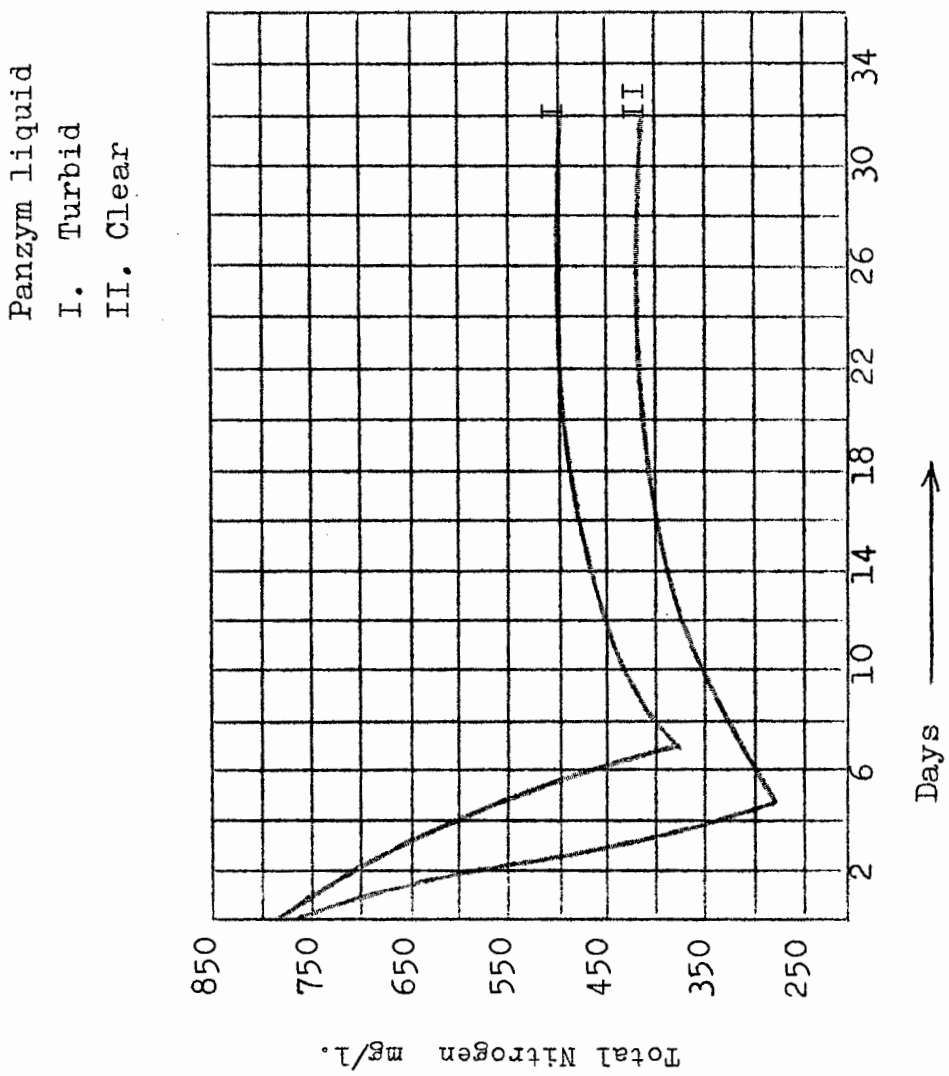
Graph No. 3.

Change in Total Nitrogen during
 Fermentation of two Samples of Must
 (i.e. Turbid and Clear)
untreated, Original.



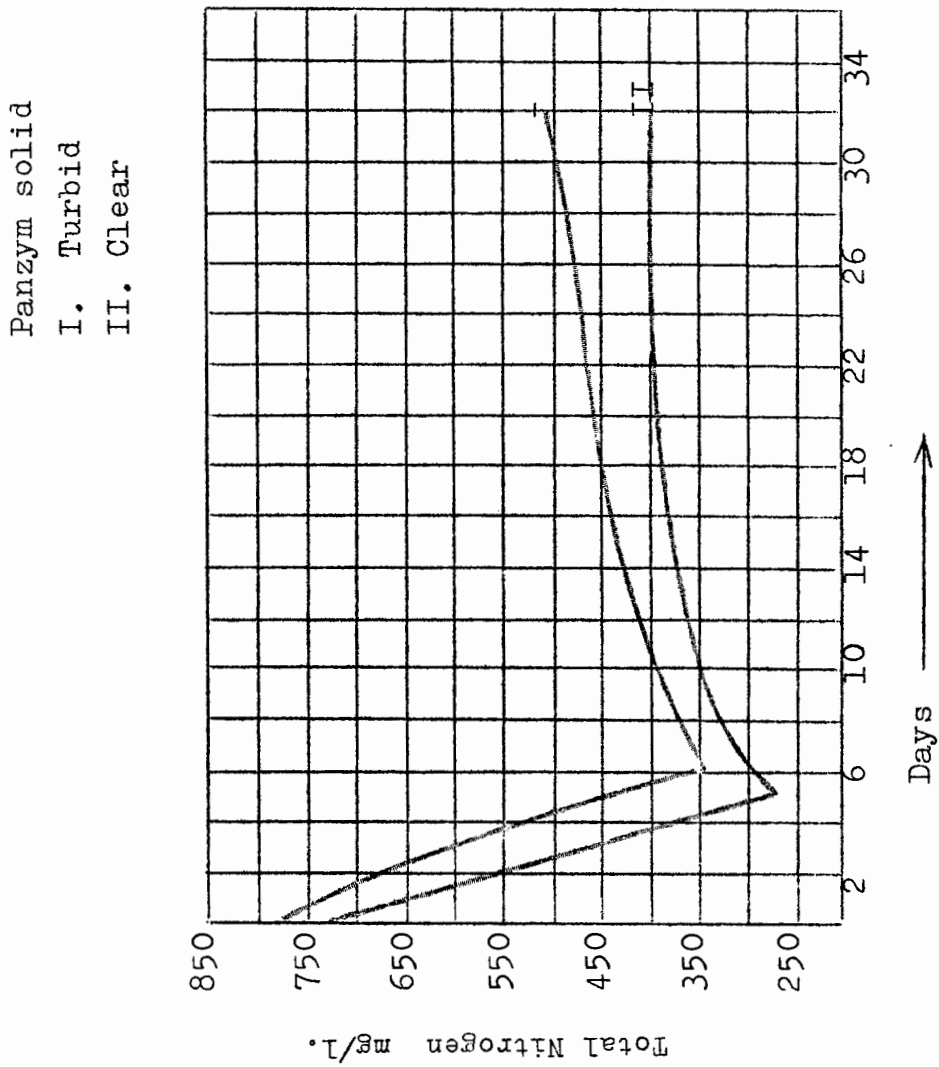
Graph No. 4.

Change in Total Nitrogen during
 Fermentation of two Samples of Must
 (i.e. Turbid and Clear)
treated with Panzym Liquid.



Graph No. 5.

Change in Total Nitrogen during
Fermentation of two Samples of Must
(i.e. Turbid and Clear)
treated with Panzym Solid.



On studying Graphs Nos. 3, 4 and 5 it becomes clear that the bulk of the fermentation was completed within ten days. During the early stages of fermentation the yeasts withdraw different substances from the substrate especially amino acids, which they require for their own synthesis. The amino nitrogen graph runs practically parallel to the graph describing the change in the total nitrogen concentration, i.e. the withdrawal from and delivery to the substrate of amino acids by the yeasts takes place proportionately to the fall and rise of total nitrogen.

The first qualitative determinations of the amino acids were carried out in an effort to establish the occurrence, disappearance and reappearance of amino acids. On the days mentioned the amino acids were determined qualitatively unidimensionally by paper electrophoresis. The results of these investigations are supplied in Tables Nos. X, XI and XII, the individual amino acids being shown as numbers according to the key supplied at the head of each table.

TABLE X.

AMINO ACIDS FOUND (UNI-Dimensionally BY PAPERElectrophoresis).

- | | | | |
|----------------------|-----------------|-------------------|-------------------|
| 1. Glycine | 6. Threonine | 11. Phenylalanine | 16. Glutamic acid |
| 2. Alanine | 7. Valine | 12. Tyrosine | 17. Arginine |
| 3. Serine | 8. Methionine | 13. Proline | 18. Lysine |
| 4. Cystine | 9. Leucine | 14. Aspartic acid | 19. Ornithine |
| 5. Aminobutyric acid | 10. Iso-leucine | 15. Asparagine | 20. Histidine |

Number of Days after Crushing	1. Turbid Original					2. Clear Original					3. Panzym Turbid Liquid					4. Panzym Clear Liquid					5. Panzym Turbid Solid					6. Panzym Clear Solid									
	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5
0	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5
	-	-	-	9	10	-	7	-	9	10	6	-	-	9	10	-	7	-	9	10	6	7	-	9	10	-	7	-	9	10	-	7	-	9	10
	11	-	-	14	-	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-
	16	-	-	-	20	16	-	-	-	-	16	-	-	-	-	16	17	-	-	-	16	-	-	-	-	16	17	-	-	-	16	17	-	-	-
3	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5
	-	7	-	9	10	-	7	-	9	10	-	7	-	9	10	-	7	-	9	10	-	7	-	9	10	-	7	-	9	10	-	7	-	9	10
	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-
	16	-	-	-	20	16	-	-	-	-	16	-	-	-	-	16	-	-	-	-	16	-	-	-	-	16	-	-	-	-	16	-	-	-	-
6	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5
	-	7	-	9	10	-	7	-	9	10	-	7	-	9	10	-	7	-	9	10	-	-	-	9	10	-	7	-	9	10	-	7	-	9	10
	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-
	16	-	-	-	-	16	-	-	-	-	16	-	-	-	-	16	-	-	-	-	16	-	-	-	-	16	-	-	-	-	16	-	-	-	-
10	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	-	-	2	-	-	5	-	2	-	-	5
	-	7	-	9	10	-	7	-	9	10	-	7	-	9	10	-	7	-	9	10	-	7	-	9	10	-	7	-	9	10	-	7	-	9	10
	11	-	-	-	-	11	-	-	14	-	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-	11	-	-	-	-
	16	-	-	-	-	16	-	-	-	-	16	-	-	-	-	16	-	-	-	-	16	-	-	-	-	16	-	-	-	-	16	-	-	-	-
13	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5
	-	7	-	9	10	6	7	-	9	10	6	7	-	9	10	6	7	-	9	10	-	7	-	9	10	-	7	-	9	10	-	7	-	9	10
	11	-	13	-	-	11	-	13	-	-	11	-	13	-	-	11	-	13	14	-	11	-	13	-	-	11	-	13	-	-	11	-	13	-	-
	16	17	-	-	20	16	17	-	-	20	16	-	-	-	20	16	17	-	-	20	16	-	-	-	20	16	-	-	-	20	16	-	-	-	20
20	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5
	6	7	-	9	10	6	7	-	9	10	6	7	-	9	10	6	7	-	9	10	-	7	-	9	10	6	7	-	9	10	6	7	-	9	10
	11	-	13	-	-	11	-	13	14	-	11	-	13	-	-	11	-	13	14	15	11	-	13	-	-	11	-	13	14	15	11	-	13	14	15
	16	17	-	-	20	16	17	-	-	20	16	17	-	-	20	16	17	-	-	20	16	-	-	-	20	16	17	-	-	20	16	17	-	-	20
32	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5
	6	7	8	9	10	6	7	-	9	10	6	7	-	9	10	6	7	8	9	10	-	7	8	9	10	6	7	-	9	10	6	7	-	9	10
	11	-	13	14	-	11	-	13	14	-	11	-	13	-	-	11	-	13	14	-	11	-	13	-	-	11	-	13	14	15	11	-	13	14	15
	16	17	-	-	20	16	17	-	-	20	16	17	-	-	20	16	17	-	-	20	16	-	-	-	20	16	17	-	-	20	16	17	-	-	20

For the qualitative uni-dimensional determination of the amino acids paper electrophoresis was employed. The samples of musts and wines were subjected to a two-hour electrophoresis according to Wieland and Pfleiderer. The buffer solution consisted of formic acid : acetic acid : water (pH 1.9). The potential difference was adjusted to 1800 volt at 20-30 mA, and the temperature at -2°C . The must or wine is stroked onto the paper by means of a micropipette, the stripe being approximately 25 cm. in length. The total volume of material to be separated amounting to 0.3 ml.

As is evident from the tables seven to eight amino acids were discernable in must prior to fermentation with alanine, aminobutyric acid, leucine and isoleucine, phenylalanine and glutamic acid as typical representatives. These amino acids were continuously to be found in the chromatograms of samples withdrawn during the earlier stages of fermentation. Once the fermentation was completed the presence of proline became apparent. The number of discernible amino acids had also increased after fermentation, nine to thirteen now being distinguishable. After 32 days eleven to thirteen amino acids were recognisable in uni-dimensional pherograms.

In the meantime a two-dimensional method for the determination of the amino acids had been worked out. A sample of must or wine corresponding to 0.5 mg. of amino acids was spotted onto the paper as previously described. After an electrophoretic separation lasting two hours the paper was dried, turned through 90° and developed for twelve hours in the second dimension using phenol : water (7:3) as mobile phase. Comparison of uni- and two-dimensional chromatograms indicated marked differences. Whilst in uni-dimensional chromatograms the presence of only eleven to fifteen amino acids could be ascertained, the two-dimensional chromatograms showed from sixteen to eighteen amino acids. It thus becomes clear that in a uni-dimensional chromatogram certain amino acids overlap on one and the same position and that such amino acids are separated by a two-dimensional method. A true representation of the amino acids present could thus only be found in a two-dimensional chromatogram.

TABLE XI.

DIFFERENCES BETWEEN UNI- AND TWO-DIMENSIONAL CHROMATOGRAPHY.

Number of Days after Crushing	1. Turbid Original					2. Clear Original					3. Panzym Turbid Liquid					4. Panzym Clear Liquid					5. Panzym Turbid Solid					6. Panzym Clear Solid									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30					
	<u>UNI-DIMENSIONAL</u>																																		
32	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5	-	2	-	-	5
	6	7	8	9	10	6	7	-	9	10	6	7	-	9	10	6	7	8	9	10	6	7	-	9	10	6	7	-	9	10	6	7	-	9	10
	11	-	13	14	-	11	-	13	14	-	11	-	13	-	-	11	-	13	14	-	11	-	13	14	15	11	-	13	14	15	11	-	13	14	15
	16	17	-	-	20	16	17	-	-	20	16	17	-	-	20	16	17	-	-	20	16	17	-	-	20	16	17	-	-	20	16	17	-	-	20
	<u>TWO-DIMENSIONAL</u>																																		
32	-	2	-	4	5	1	2	-	4	5	1	2	3	4	5	1	2	-	-	5	1	2	-	4	5	-	2	-	4	5	-	2	-	4	5
	6	7	8	9	10	6	7	8	9	10	6	7	8	9	10	6	7	8	9	10	6	7	8	9	10	6	7	8	9	10	6	7	8	9	10
	11	12	13	14	15	11	12	13	14	15	11	12	13	14	15	11	12	13	14	15	11	12	13	14	15	11	12	13	14	15	11	12	13	14	15
	16	17	-	19	20	16	17	-	19	20	16	17	18	-	20	16	17	-	-	20	16	17	-	-	20	16	17	-	-	20	16	17	-	-	20

In order to shed light on these differences another fermentation study was undertaken. In this experiment the individual amino acids were not only qualitatively indicated but also quantitatively determined.

This quantitative method of determination of the amino acids was applied in the course of this new experiment. In the following table a summary and comparison of the two-dimensional determination and the quantitative analysis of the amino acids are given.

All amino acids, excepting proline, were determined as alanine, the proline being determined as such and referred to alanine by multiplication with the factor 0.775.

TABLE XII

QUANTITATIVE DETERMINATION OF THE AMINO ACIDS, REFERRED TO ALANINE.

1. Turbid Original					2. Clear Original					3. Panzym Turbid Liquid					4. Panzym Clear Liquid					5. Panzym Turbid Solid					6. Panzym Clear Solid				
1	2	-	4	5	1	2	-	4	5	1	2	3	4	5	1	2	-	-	5	1	2	-	4	5	-	2	-	4	5
6	7	8	9	10	6	7	8	9	10	6	7	8	9	10	6	7	8	9	10	6	7	8	9	10	6	7	8	9	10
11	12	13	14	15	11	12	13	14	15	11	12	13	14	15	11	12	13	14	15	11	12	13	14	15	11	12	13	14	15
16	17	-	19	20	16	17	-	19	20	16	17	-	19	20	16	17	-	-	20	16	17	-	-	20	16	17	-	-	20

Proline is estimated, using the Graph No. Page and referred to Alanine (Factor 0.775).

	mg/l.	mg/l.	mg/l.	mg/l.	mg/l.	mg/l.
2. Alanine	44	63	27	47	58	94
3. Serine	-	-	68	84	-	-
6. Threonine	77	40	33	-	63	53
9. Leucine and 10. Iso-leucine	80	⊗	105	52	80	49
13. Proline	1225	1206	1062	1316	998	1163
16. Glutamic acid	56	43	46	76	∅	∅
17. Arginine	342	346	317	410	412	502
11. Phenylalanine 18. Lysine 19. Ornithine and 20. Histidine	102	55	58	65	112	155
1. Glycine 4. Cystine 5. Aminobutyric acid 7. Valine 8. Methionine 12. Tyrosine 14. Aspartic acid and 15. Asparagine	162	164	155	188	176	108

⊗ Determined with Alanine

∅ Determined with Leucine and Iso-Leucine

The quantitative determination of the amino acids rendered a picture somewhat different from the general conception. In order of concentration proline and arginine are very prominent. In considerably smaller concentrations leucine, isoleucine, glutamic acid, serine, alanine and threonine are found, whilst the remainder of the amino acids appeared in quantities that could often be regarded as mere traces. In order to reach a numerical representation of the different amino acids it was often necessary to determine them in groups as can be seen from the tables.

It is known that the enzyme aspartase can form aspartic acid from fumaric acid and ammonia. Furthermore it is also a well-established fact that the glutamic acid dehydrogenase through a reductive amination can change the α -ketoglutaric acid to glutamic acid. Glutamic and aspartic acids are regarded as the precursors in the amino acid synthesis. From the glutamic acid via the glutamines the paths leading to the formation of proline are well defined. The explanation for the formation of arginine which next to proline forms the single component showing the highest concentration among the twenty amino acids found in wine, seems to be much more complicated.

During the 1957 vintage Riesling grapes in different stages of ripening were examined. When the must showed 32° Oechsle, a stage which could arbitrarily be designated as the start of the ripening process, only arginine was found to be present. Eight days later alanine and glutamic acid could be observed. The aromatic systems, i.e. phenylalanine, tyrosine and proline only occurred after a definite amount of sugar had been formed in the berries, i.e. at 60° Oechsle. After this stage the amino acids increased rapidly so that ten days later seventeen amino acids could be registered.

TABLE XIII.

TWO-DIMENSIONAL ANALYSIS OF RIESLING GRAPES IN DIFFERENT STAGES OF DEVELOPMENT.

Date	Degrees Oechsle	Occurrence of Amino Acids										Remarks									
		1. Glycine	2. Alanine	3. Serine	4. Cystine	5. Aminobutyric acid	6. Threonine	7. Valine	8. Methionine	9. Leucine	10. Iso-leucine	11. Phenylalanine	12. Tyrosine	13. Proline	14. Aspartic acid	15. Asparagine	16. Glutamic acid	17. Arginine	18. Lysine	19. Ornithine	20. Histidine
4.8.1957	18																				
12.8.1957	26.5																				
22.8.1957	32.5																				Occurrence of Arginine
1.9.1957	39.0						2	16	17												Occurrence of Alanine and Glutamic acid
12.9.1957	45.0						-	2	3	4	-										
							6	-	8	-	-										
							-	12	-	-	-										
							16	17	-	-	-										
18.9.1957	59.5						-	2	3	4	-										Occurrence of Aromatic Systems: Phenylalanine and Tyrosine
							6	-	8	-	-										Occurrence of Proline
							11	12	13	-	-										
							16	17	-	-	-										
1.10.1957	60.5						-	2	3	4	-										do.
							6	7	8	9	10										
							11	12	13	14	-										
							16	17	-	-	-										
11.10.1957	67.0						-	2	3	4	5										do.
							6	7	8	9	10										
							11	12	13	14	-										
							16	17	-	19	20										
22.10.1957	*62.0						-	2	3	4	5										
							6	7	8	9	10										
							11	12	13	14	-										
							16	17	-	19	20										

* After heavy rainfall

The next experiment was carried out with Waltham Cross table grapes in an endeavour to ascertain to what extent the total amino acid content and individual amino acid concentration undergo changes during and after fermentation. Simultaneous analyses were carried out for total nitrogen. From the fermentation graph it can be seen that the total nitrogen concentration diminished in the same proportion as the total amino acid concentration. Also in this instance did proline and arginine prove to be the numerically most important amino acids, their presence already being registered in the fresh must. During the fermentation these two substances are utilised by the yeasts and later they are returned to the young wine.

TABLE XIV TOTAL NITROGEN AND TOTAL AMINO ACIDS (REFERRED TO ALANINE) AT DIFFERENT STAGES DURING THE FERMENTATION OF A WALTHAM CROSS MUST.

Days after Fermentation	Analysis of Amino Acids	Total Nitrogen in Clear Filtrate mg/l.	Amino Acids together referred to Alanine mg/l.	Alanine with Factor 0.1573 referred to Total N ₂ mg/l.	Key Number of Amino Acids Found	Amino Acids	Amino Acids found mg/l. referred to Alanine					
							I	II	III	IV	V	VI
0	I	759	3663	567	2	Alanine	96	63	x	x	27	62
7		584	2648	432	3	Serine	63	45	46 ^{xx}	12 ^{xx}	63	72
10	II	490	2353	370	6	Threonine	42	29	xx	xx	23	55
12		467	2226	350	9, 10	Leucine and Iso-leucine	64	57	x	x	x	57
14	III	391	1952	307	13	Proline	1205	755	687	523	748	963
16		304	1514	238	16	Glutamic Acid	44	27	66 ^x	50 ^x	45 ^x	24
18	IV	239	1049	165	17	Arginine	1316	1016	885	340	402	526
23		219	1081	170	11, 18, 19 and 20	Phenylalanine, Lysine, Ornithine and Histidine	257	112	82	57	88	152
30	V		1558	245								
36		339	1781	280	1, 4, 5, 7, 8, 12, 14 and 15	Glycine, Cystine, Aminobutyric Acid, Valine, Methionine, Tyrosine, Aspartic Acid and Asparagine	503	283	177	103	216	302
50	VI	397	2200	346								
Total							3590	2387	1943	1085	1612	2213
Total of previous Table							3663	2353	1952	1049	1558	2200
Difference							-173	+34	-9	+36	+54	+13

(Also note Graphs for total nitrogen and Amino Acids in terms of total nitrogen)

Under

- I All Amino Acids Nos. 1-20 were present.
- II Absent: 4. Cystine, 8. Methionine, 12 Tyrosine and 18. Lysine.
- III Absent: 1. Glycine, 4. Cystine, 8. Methionine, 12. Tyrosine, 15. Asparagine and 18. Lysine.
- IV Absent: 1. Glycine, 4. Cystine, 12. Tyrosine, 15. Asparagine and 18. Lysine.
- V Absent: 1. Glycine, 4. Cystine, 12. Tyrosine and 15. Asparagine.
- VI Absent: 1. Glycine, 4. Cystine and 12. Tyrosine.

x These Amino Acids are determined together

xx These Amino Acids are determined together

Graph No. 6. The Relationship between Total and Amino Nitrogen.

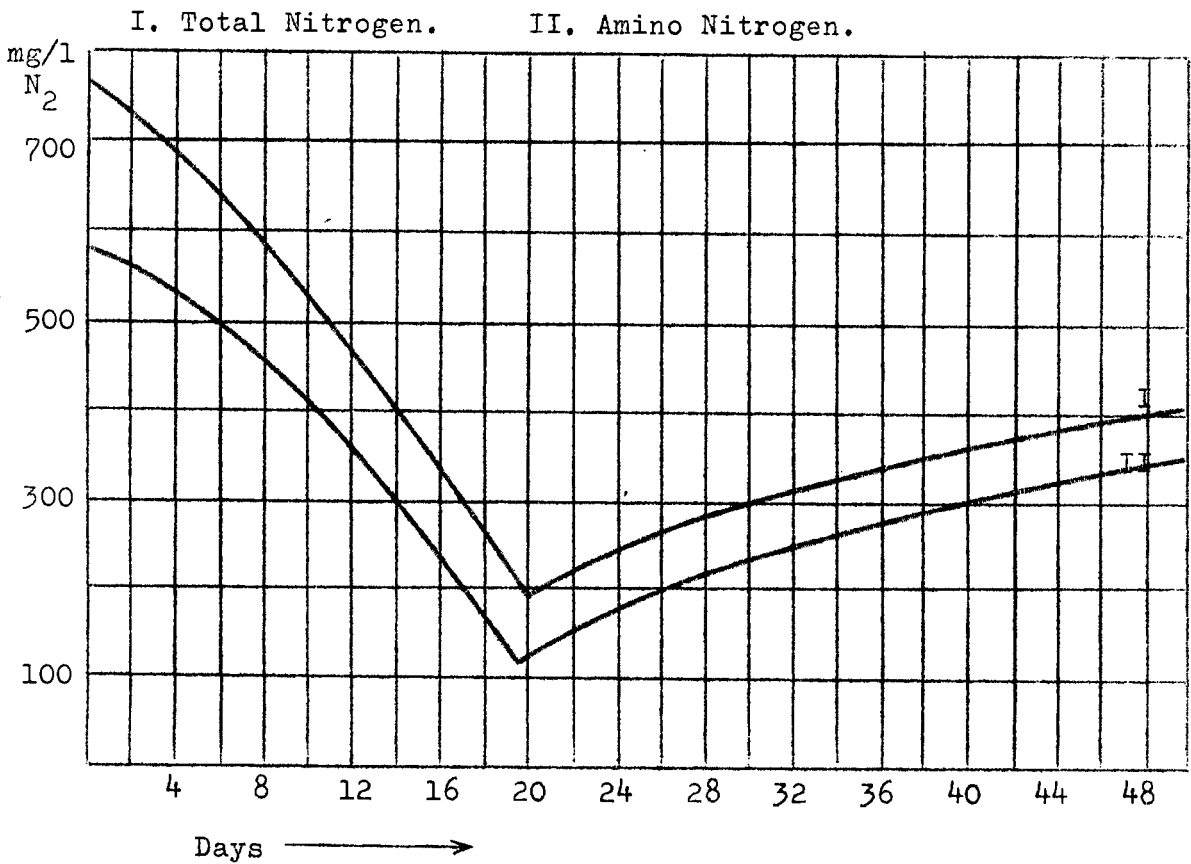





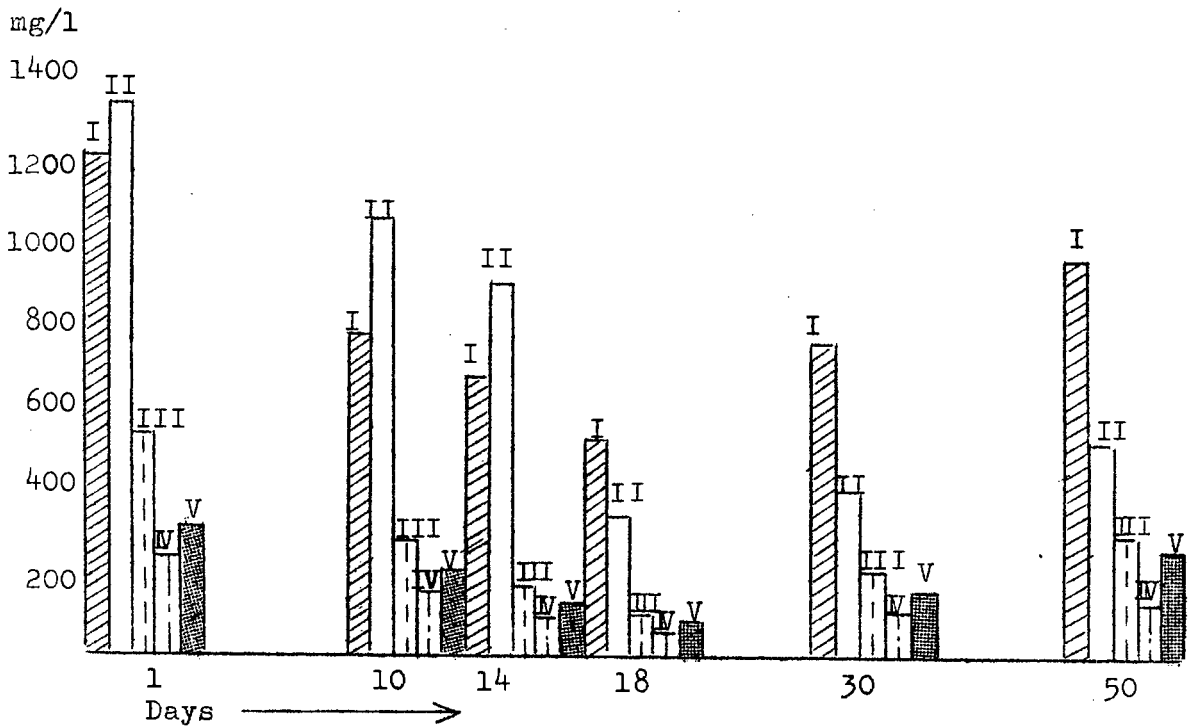


Diagram II. Status of Amino Acids in relation to Data supplied in Graph No. 6.

-  I. Proline.
-  II. Arginine.
-  III. Glycine, cysteine, aminobutyric acid, valine, methionine, tyrosine, aspartic acid, asparagine.
-  IV. Phenylalanine, lysine, ornithine, histidine.
-  V. Alanine, serine, threonine, leucine, iso-leucine and glutamic acid.



S U M M A R Y.

A method for the qualitative and quantitative determination of amino acids in musts and wines is suggested.

The qualitative determination is based on a two-dimensional method. Separation in the first direction is effected by electrophoresis, the interfering substances being left in the vicinity of the starting point. For the development in the second direction the ordinary chromatography is employed, separating the still unseparated amino acids.

The distances travelled by the different amino acids during electrophoresis are referred to the distance travelled by alanine. These R_{Alanine} values together with the R_f -values obtained from the upward development with phenol : water (7:3) as mobile phase, are furnished.

According to the abovementioned methods, the quantitative determination of amino acids were also carried out. When the amino acids occur in minute quantities they are grouped together so as to facilitate their quantitative determination. All amino acids, barring proline, are estimated as alanine, proline being estimated as such.

Examinations of musts to which different enzyme preparations had been added were carried through.

During fermentation the yeasts withdraw amino acids from the substrate. The appearance and disappearance of the individual amino acids are shown.

In two-dimensional chromatograms it was shown that a must could contain up to twenty different amino acids.

During the ripening of the grape berry arginine was the first to appear, followed somewhat later by alanine and glutamic acid. Probably the arginine has a buffering influence on the acids which develop during the early stages of ripening.

Once a definite stage in the ripening is reached the aromatic amino acids, i.e. phenylalanine, tyrosine and proline can be observed.

It may be assumed that the proline and arginine are of major importance during the fermentation. The remaining amino acids appear in quantities ranging from small to minute.

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A P P E N D I X I.

AMINO ACIDS.

Fig. I.

Pherogram of Amino Acids.

Developed for two Hours at -2°C ,
20 mA and 1850 V.

Spray: Ninhydrin solution.

Lys.

Orn., Ser., Gly.

Arg. Hist.

Phenyl., Leuc., Iso-leuc.

Am.but., Asp., Ala.

Pro.

Meth.

Cystine, Val., Tyr.

Glut., Asp. acid

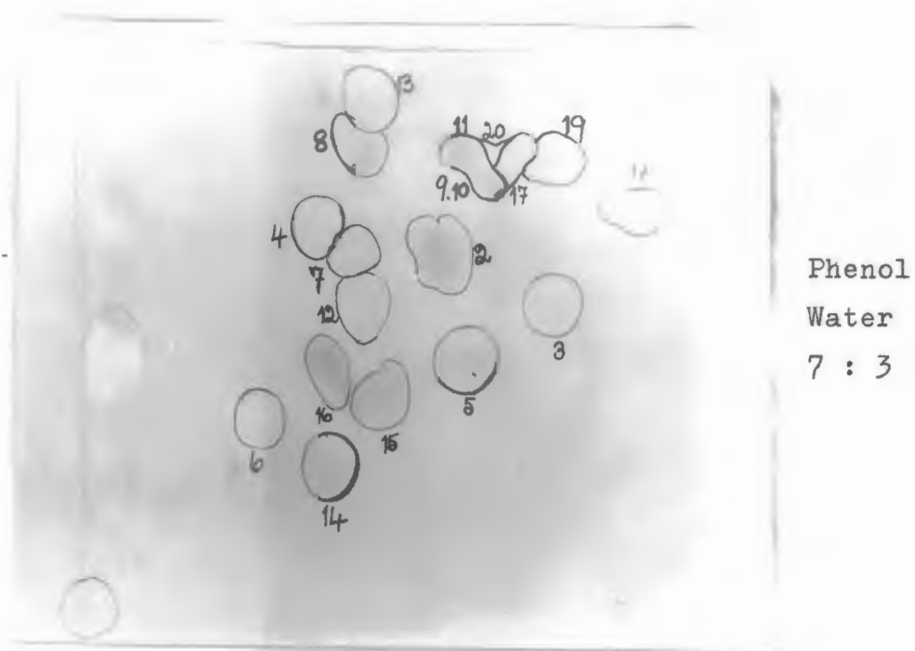
Threo.

Starting point



Fig. II.

Two-Dimensional Separation of the Amino Acids.
(See Diagram I, page 26).



Starting point

Electrophoresis

-2°C 1850 V. 24 mA.
2 Hours.

Fig. III.

The similar Pherogram to that shown in Fig. I first sprayed with 2% Ethyldiamine hydrate solution, dried and subsequently sprayed with 0.02% Ninhydrin solution.

Amino acids

Starting point

Sugar



A QUALITATIVE AND QUANTITATIVE DETERMINATION
OF THE AROMA SUBSTANCES, ESPECIALLY ESTERS,
ALDEHYDES AND KETONES IN MUSTS AND WINES.

LITERATURE - GENERAL.

Guenther¹⁾ in his authoritative work "The Essential Oils" points out that earlier works referred to the fruit ethers or ethereal oils, which result when different plant materials are subjected to a steam distillation, as volatile oils.

Haagen-Smit²⁾ points out that a microscopic examination of plants presenting a typical smell, proves that certain cells or openings in the plant texture are filled with oily globules. These globules react in a positive manner to fat stains. Two characteristics distinguish these oils from the ordinary fats, i.e. their characteristic oil and their volatility. These oils are designated as the so-called essence carriers of the plant. This then may be presumed to be the source of the term essential oils as generally applied in countries of Anglo-saxon origin.

In many instances the cells forming these oils are situated in the epidermis. Often a gentle touch suffices to evoke the typical aroma of a particular plant. In many instances the oils are located in specific plant glands.

The volatile oils are often not originally present in a plant. Sometimes they result from the hydrolysis of the glycosides. If portions of a plant possessing these glycosides are crushed, then the volatile portion of the glycoside is rendered free by certain enzymes. As examples in this respect methylsalicylate and benzaldehyde may be mentioned as forming the characteristic components of wintergreen and peach kernel oils respectively.

In wine a difference is drawn between primary grape bouquet and secondary grape bouquet. The primary bouquet substances are derived from the grapes whilst the secondary bouquet substances originate during the fermentation. To this must be added the bouquet substances formed during maturation, i.e. the maturation bouquet.

When the definition of the essential oils as supplied in the first paragraph is once more considered, it immediately becomes apparent that this group can include many and varied substances in spite of the fact that their characteristic volatility does appreciably lessen their number.

Haagen-Smit³⁾ contends that these substances all exhibit a boiling point of less than 300°C, i.e. only under exceptional circumstances can they have more than twenty carbon atoms to the molecule. Thus other high-molecular fat-soluble compounds like sterols are not classified as ethereal oils. These substances also do not contain a large number of hydroxyl groups, thus ruling out sugars, glucosides and carbohydrates. Strongly ionising compounds such as salts, acids and alkalies are also not regarded as belonging to this group. Although the definition limits the number of compounds belonging to this class, a large number of components displaying marked differences, are included. Both aromatic and aliphatic compounds with their derivatives are included. Many of the aromatic substances include in their molecules elements like oxygen, nitrogen and in some instances even sulphur. Esters, aldehydes, ketones, acids, alcohols, lactones, amines, mercaptans, etc., all find representation in this group.

It would be impracticable for these substances to be classified according to their chemical properties. It is more advantageous to arrange them according to biochemical origin. According to such a classification, straight-chain compounds, irrespective of their stage of oxidation, are grouped together as aldehydes, ketones, esters, alcohols and acids.

1. Straight-chain compounds as already indicated are very widely dispersed among the plant aroma substances. They form the bulk of the wine bouquet.
2. The next group has a benzene ring in the molecule. These substances are of great importance to the producers of industrial essences. Benzaldehyde and vanilline as the characteristic components of the oils of bitter almonds and vanilla pods respectively, are typical representatives of this group. Some of the compounds belonging to this group have been detected in wine.
3. In the third group approximately 500 compounds can be grouped together, each one possessing as its basic molecular configuration, a branched C-5 chain. To these groups belong the terpenes which form the characteristic component of many fruit ethers, e.g. oils of turpentine and peppermint.

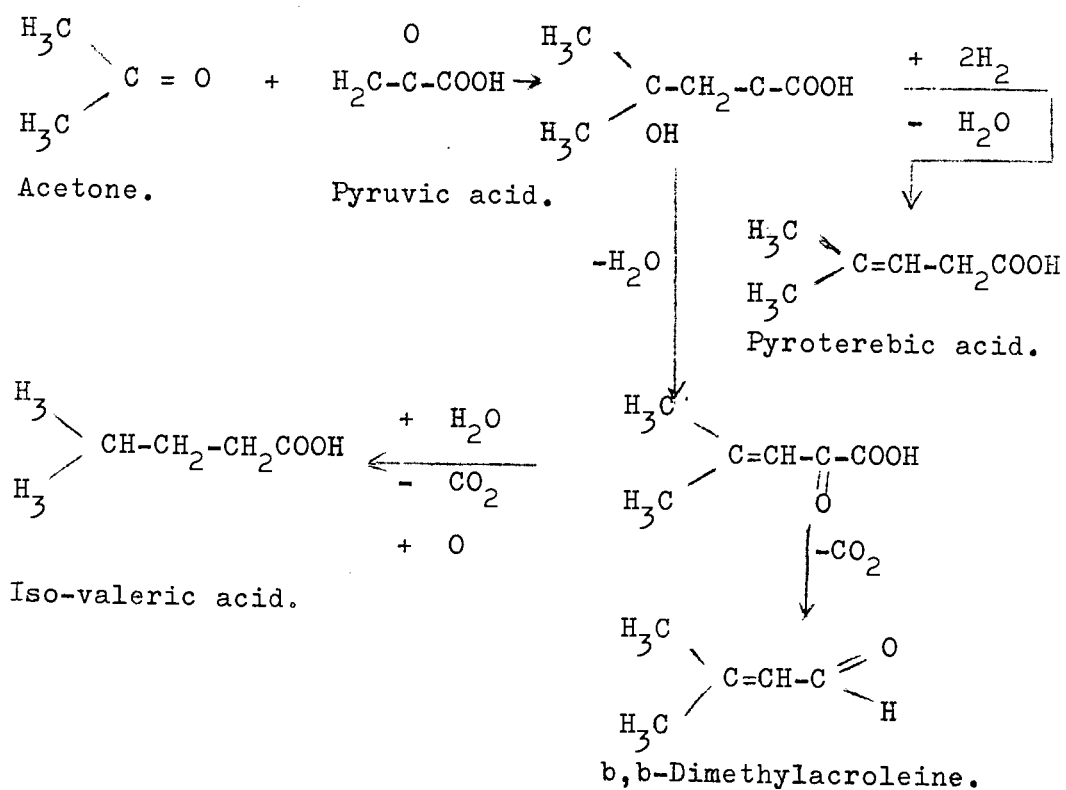
4. In.....

4. In this group we find miscellaneous substances not belonging to any of the abovementioned three. Indol, a heterocyclic compound having both the elements nitrogen and oxygen incorporated in its molecule, belongs to this group.

Because of their resemblance to fatty acids the straight-chain hydrocarbons are generally held to originate from the fatty acid metabolism, whilst the benzene and propylbenzene derivatives are regarded as products of the carbohydrate metabolism.

Although this work occupies itself exclusively with components included in the first of the above groups, it would perhaps not be completely amiss to make brief mention of the terpenes. Generally speaking these compounds are the most important constituents of the fruit ethers. In wine they are of lesser importance. According to the hypothesis propounded by Guenther⁴⁾ the terpenes are synthesised from precursors possessing a keto-group and whose presence in wine is already an established fact.

It has already been said that the terpenes have the basic configuration of a branched C-5 chain and furthermore it can be assumed that isoprene (C₅H₈) is an important precursor of the terpenes. This substance is formed according to the generally accepted hypothesis of the terpene formation as a result of a condensation of acetone and pyruvic acid.



Isoamyl alcohol is formed through the degradation of the carbohydrates, the protein bodies and amino acids, e.g. leucine. From leucine, isovaleric acid and pyroterebic acids are readily obtained. Haagen-Smit⁵⁾ points out that they are also found together among the aroma substances of several plants, e.g. *Kalotropis procera*.

According to the generally accepted viewpoint terpenes have their origin in the carbohydrate degradation. From already established biochemical evidence it can be stated that the ethereal oils are formed during the vital plant processes and that they are very intimately associated with plant physiology.

The growing plant absorbs various substances from its surroundings and transforms them within the plant body. Many of these substances are stored within the plant. This transformation of absorbed substances and their subsequent storage within the plant is accompanied by many and varied reactions. A portion of newly-formed substances are oxidised in order to render to the plant its required energy. The equilibrium between this degradation and biosynthesis determines the growth of a plant. Many of these substances formed by the plant remain in continuous flux, whilst many others apparently fulfil no further function during the life of the plant.

Cellulose is incorporated in the cell wall in order to give the plant its required stability, other substances like starch are stored as potential energy, later to be released if and when required by the plant. To attach a sharply defined function to all plant substances is hardly possible. This becomes doubly complicated in the case of the aroma substances alkaloids, anthocyanins, tannins and resins. Here the question arises as to whether the aroma substances are of any particular value to the plant. Is their presence purely incidental or even superfluous? In general it can be said that during the life cycle of a plant many compounds belonging to the volatile aroma-yielding substances fulfil an important function, they often function as either hydrogen acceptors or hydrogen donors. Once these substances become involved in the processes of fermentation they are dissociated from their previous purely vegetable environment.

It can rightly be said that the knowledge of individual substances remains far greater than the knowledge of their

origin.....

origin or purpose. Before entering into a discussion on the aroma substances in musts and wines it would perhaps be proper to present a short summary of such substances already indicated in other plants.

Power and Chestnut^{6 & 7)} detected free furfural and acetaldehyde in the steam distillate of ripe apples. As esterified substances they found methyl alcohol, amyl alcohol, acetic and caproic acids. In the distillate derived from the peels of ripe apples they found geraniol. Howard^{8 & 9)} on the other hand, lists the following as constituents of the apple aroma: acetaldehyde, n-hexylaldehyde, methyl alcohol, ethyl alcohol, n-butyl alcohol, n-hexanol, n-propyl alcohol, ethyl acetate, ethyl formate, ethyl propionate, ethyl isopropionate, isobutanol, d-2-methyl butyl alcohol, n-capronaldehyde, 2-hexanal, methyl butyraldehyde, ethyl caproate and acetone. The abovementioned alcohols constituted 92%, the aldehydes 6% and the esters 2% of the volatile oils.

Nelson and Curl¹⁰⁾ distilled 94 litres of cherry juice under vacuum and subsequently the volume was decreased to 500 ml. under vacuum. They found methyl alcohol, ethyl alcohol, benzaldehyde and traces of geraniol.

Hall and Wilson¹¹⁾ found that the juice from Valencia oranges contained acetaldehyde, acetone, ethyl alcohol, acetic and caprylic acids, geraniol and amyl alcohol, whilst the orange peels contained citral and decylaldehyde.

Comprehensive work on the aroma substances of pineapples was done by Kirchner¹²⁾ and Haagen-Smit, Kirchner, Prater and Deasy^{13 & 14)}. They worked with 2896 Kg. of peeled pineapples under vacuum at 40°C. The distillate after passing through a cooler was received in a series of traps ranging in temperature from -20° to -180°C. They ascertained that the aroma substances consisted primarily of esters. The separated esters were hydrolysed, the alcohol fraction separated into individual components by means of the 3,5-dinitrobenzyl esters and the acid fraction into its various representatives by means of the phenylphenacyl esters. They made the interesting observation that barring ethyl acetate the esters of the winter fruit consisted exclusively of methyl esters, whilst summer fruit consisted mainly of ethyl esters. These authors established the presence of the sulphur-containing ester methyl-b-methyl thiopropionate ($\text{CH}_3\text{SCH}_2\text{COOCH}_3$).

In winter fruit ethyl acetate, acetaldehyde, methyl isocaproate, methyl isovalerate, methyl n-valerate, methyl caprylate and methyl-b-methyl thiopropionate were found. In summer fruit the presence of the following compounds was observed: ethyl acetate, ethyl alcohol, acetaldehyde, ethyl isovalerate, methyl n-valerate, propyl ketone, ethyl acrylate, methyl n-caproate.

Coppens and Hoejenbos^{15 & 16)} investigated the volatile compounds of raspberries and strawberries. In the former caproic and benzoic acids, diacetyl, ethyl alcohol, ethyl acetate, isoamyl alcohol, benzaldehyde and phenylethyl alcohol were found. In the latter acetic, caproic, cinnamic and benzoic acids, as well as ethyl alcohol could be observed. Furthermore these authors found in strawberries either the butyric or acetic acid ester of either ethyl or isopropyl alcohol and isoamyl caproate, dl-terpineol, l-borneol and dl-isophenyl alcohol.

With regard to the aroma substances in vegetables the following may briefly be discussed. Richter¹⁷⁾ found butyric and palmitic acids and in all probability esters of acetic and formic acids in carrot seed oil. Pinene, l-limonene and daucol were also found to be present in this oil. In oil derived from celery seeds he found l-limonene and palmitic acid. In garlic and onions he found allyl sulphide $(C_3H_5)_2S_2$ and compounds of the following formulae: $C_3H_5-S-S-C_3H_5$ and $C_3H_5-S-S-S-C_3H_5$.

Takey and Ono¹⁸⁾ subjected cucumber to a steam distillation. This distillate was afterwards extracted with ether and the presence of the oil 2,6-nonadienal was ascertained.

The aroma substances of the abovementioned fruits and vegetables are thus largely composed of volatile compounds. When the number of plants, the aroma constituents of which have already been investigated, is compared to those which have as yet received no attention, the lack of knowledge in this field immediately becomes apparent. This can chiefly be explained as being due to the small concentrations in which such substances are observed as well as to their pronounced volatility.

Before the microchemical paper chromatography existed the amount of raw material necessary to render say 1 gm. of volatile oil, often assumed enormous proportions. Once the

fractionation.....

fractionation and identification of such a sample is undertaken the "trace" fractions are often of necessity neglected. Often such substances of seemingly negligible concentration play an important part in a "balanced aroma". This then is also the explanation why many of the earlier authors did not occupy themselves with such micro-compounds.

Although considerably simplified the modern micro-chemical analysis of the aroma substances still remains enormously difficult. The hydrolysis of an ester mixture might indicate which alcohols and acids are present, but the individual combination usually remains obscure.

Many of the earlier investigations were carried out before satisfactory reagents for the identification existed. Often earlier workers were forced to employ the olfactory sense as the only means of identification. Nevertheless, it cannot be denied that the sense of smell forms a powerful ally in the positive identification of an unknown substance.

Fundamental knowledge of the aroma substances in wines is of importance to the wine farmer, merchant and expert, rendering insight into the influence of different operational techniques on the final product.

SUMMARY OF LITERATURE ON THE AROMA SUBSTANCES IN GRAPES, GRAPE-JUICE, WINE AND BRANDY.

The substances which render a specific bouquet or aroma to individual varieties like Riesling, Traminer, Sylvaner, Müller-Thurgau, Muscatel, etc. are not limited in their occurrence to the berry husk alone, but are often distributed through the entire berry fabric (compare Babo and Mach¹⁹). Von der Heide²¹) refers to these substances as primary grape bouquets, whilst Müller-Thurgau^{21 & 22}) defines them as "aromatic" substances.

The estimation of the aroma substances in wines has always been a fascinating study. It has for many years been occupying the time of various investigators. Even as early as 1836 Justus von Liebig²³) deemed it fit to occupy some of his time with this interesting chapter of the eno-chemistry.

Cognac analyses are reported on by Ordonneau²⁴⁾ in 1887 and by Morin²⁵⁾ in 1888. They found that the higher alcohols in the brandies examined by them consisted primarily of butyl and amyl alcohols, whilst the occurrence of propyl alcohol although in much lesser quantity, was nearly always registered. Furthermore, they found acetic, propionic, butyric and caproic acids free or in esterified form.

Lauric acid was found by Grossfeld and Miermeister²⁶⁾ in fusel oil. Webb et al²⁷⁾ contended that the esterified acids in brandy range from caproic to palmitic acid. The latter also found that apart from amyl, butyl and propyl alcohols, hexyl and heptyl alcohols were also present.

In the aldehyde series in brandy Tillmans²⁸⁾ registered the presence of acetaldehyde. The presence of formaldehyde and some of the higher homologues was also indicated at times. According to Büttner²⁹⁾ the presence of the following substances other than those already cited, could be registered: diacetyl, terpenes, isobutylene glycol, acetoin, acetone and acrolein.

According to Windisch^{30 & 31)} the amyl esters of all aliphatic acids ($C_1 - C_{10}$) barring enanthic acid (C_7) are present in brandy, whilst as free acids only the $C_1 - C_5$ range occurs. Windisch also grouped all the aroma substances formed during fermentation together under the common grouping of "oenanthic ethers". He also contended that the typical wine bouquet (Wein Geruch) stems from these substances. According to this writer the term wine bouquet ("Wein Geruch") should be divorced from the ordinary expression "aroma".

Power and Chestnut³²⁾ found 0.2 to 2 mg/l. methyl anthranilate in the juice of various *Labrusca* varieties, whilst Sale and Wilson³³⁾ were unable to find this substance in the juice of *Vinifera* varieties. In following up the work of Waser and Mosca³⁴⁾ on the aroma substances in cherry mash, Hennig and Villforth^{35 & 36)} made a considerable contribution to the aroma substances in wine. They worked out a method whereby the aroma compounds are cold-extracted with pentane. The analysis of the individual separated compounds was carried out using generally established methods. The representatives of the different groups of aldehydes, ketones, alcohols and fatty acids found, are:-

A. ALDEHYDES AND KETONES.

Occurrence.

<u>Established.</u>	<u>Probable.</u>	<u>Possible.</u>
Acetaldehyde	Formaldehyde	Propionaldehyde
Vanilline	Methylacetyl carbinol	Higher aldehydes
Cinnamic aldehyde		Benzaldehyde
Acetone		Furfural
		Methyl ketone

B. ALCOHOLS (AS ESTER COMPONENTS).

Occurrence.

<u>Established.</u>	<u>Probable.</u>	<u>Possible.</u>
Methyl alcohol	n-Propyl alcohol	-
Ethyl alcohol	n-Heptyl alcohol	
Isopropyl alcohol	sec. Nonyl alcohol	
Isobutyl alcohol		
Isoamyl alcohol		
a-Terpineol		

C. FATTY ACIDS (AS ESTER COMPONENTS).

Occurrence.

<u>Established.</u>	<u>Probable.</u>	<u>Possible.</u>
Formic acid	Isobutyric acid	Pelargonic acid
Acetic acid	Isovaleric acid	Myristic acid
Propionic acid	Heptanoic acid	
n-Butyric acid		
n-Caproic acid		
Caprylic acid		
Capric acid		
Lauric acid		

To maintain the chronological order the American findings on this subject are given. Haagen-Smit et al.³⁷⁾ vacuum distilled 1100 litres *Vitis vinifera* (var. Zinfandel) juice at 40 - 50 mm. Hg and at 45-50°C. They extracted the distillate with ether, dried the ether extract over sodium sulphate and evaporated the ether. The 158.3 ml. ether-free remaining liquid

was.....

was fractionated, the process under normal atmospheric conditions repeated under vacuum. The following fractions were obtained:-

	<u>ml / 1,000 Kg. Fruit.</u>
Acetaldehyde	1,800
Hexanaldehyde	0,327
Ethyl alcohol	244,000
Acetylmethyl carbinol	0,013
Acetic acid	0,0053
n-Caproic acid	0,0015
n-Butyric acid	0,003
Glyoxalic acid	0,118
n-Butylphthalate	2,250
Fatty substance	0,024
Carbonyl compound	0,025
Sulphur	0,004

Kepner and Webb³⁸⁾ investigated the volatile compounds of different varieties of the *Vitis rotundifolia*. They first examined the must and found ethyl alcohol, n-butyl alcohol, phenylethyl alcohol and esters of acetic, lauric and isopropionic acids.

The presence of methyl alcohol, n-hexanal, l-hexanal and acetal was considered highly probable. During the following season they found in a wine from the same variety methyl alcohol, isoamyl alcohol, acetaldehyde, biacetyl, n-hexanal, 2-hexanal, isobutylaldehyde, ethyl acetate, caproic acid ester and a caprylic acid ester. Highly probable remained the presence of methyl-ethyl ketone and acetal. According to their experience the Flash Evaporator Technique could be more advantageously employed than the generally employed vacuum distillation. The volatile oils gained from *Vitis rotundifolia* were free from sulphur or nitrogen compounds, while the *Labrusca* varieties generally possessed an anthranilic acid ester, especially the methyl derivative.

Webb and Kepner³⁹⁾ examined four different fusel oils. These samples were vacuum-distilled in a special apparatus consisting of a 40-plate Oldersham Rectifying Column with reflux condenser. They maintained that the pleasant smelling fractions of the fusel oils are completely masked by the C₃, C₄ and C₅ alcohols.

Frey and Wegener^{40 & 41)} distilled 1,000 litres of French low wines in an endeavour to establish a general picture for the behaviour of aroma yielding substances during the brandy distillation. Paperchromatographically they ascertained that the early fractions in the distillation process primarily contain the esters of acetic and butyric acids. The propionic acid was mostly found in the free form. Valeric acid was present in very small concentrations, positive occurrence only discernable from the late middle-run fractions. Caproic acid was found to be present, both free and esterified. Higher than C_6 the acids occur in either the free or esterified form during the entire course of the distillation. The esterified acids of the middle-run are attached mainly to ethyl alcohol. The middle-run shows a larger concentration of free higher alcohols. The esters of higher alcohols are present in higher concentration during the first half of the middle-run. Higher fatty acids attached to ethyl alcohol are especially strongly represented in the "feints".

The authors whom have been cited up to this point all used large quantities of material to be examined (compare Hennig and Villforth, 360 litres and Frey and Wegener, 1,000 litres). Bayer et al^{42, 43, 44, 45, 46)} were the first to estimate the bouquet substances in wine, i.e. the esterified aliphatic and aromatic fatty acids and aldehydes, starting with 100-500 ml. of material.

The earlier work was very laborious and expensive. Furthermore, it was often impossible to gain quantitative results. The method as proposed by Bayer makes possible the analyses of musts from new crosses and high quality wines where the samples are inevitably limited. They contend that propionic and butyric acids and particularly valeric acid and aromatic carboxy acids are important constituents of quality wines. They failed to establish a simple relationship between the formic acid and higher aliphatic acid ($C_6 - C_{10}$) content of a wine and its quality. In grape juice they found predominantly formic, acetic and aliphatic ($C_6 - C_{10}$) fatty acids and to a lesser extent propionic, butyric, valeric and caproic acids and aromatic fatty acids. The qualitative values for the esters as obtained by Bayer do not seem to be in agreement with the values obtained for total volatile ester content according to Hennig⁴⁷⁾. Bayer recovered in the vicinity of one-tenth of the generally to be expected amount of volatile esters.

Bayer also found that when the SO₂-addition compounds present in wine are destroyed by the addition of alkali and subsequently extracted with ether, such wines show the presence of acet-, propion-, butyl- and valeraldehydes, hexanal and traces of higher aliphatic aldehydes. Valeraldehyde generally figures most strongly next to acetaldehyde. In his opinion the aldehydes play no major part in the establishment of a typical wine bouquet.

SUMMARY OF LITERATURE ON THE DIFFERENT METHODS OF ANALYSIS.

This summary embraces mainly the micro-chemical methods of analysis. As can be gathered from the literature review the modern micro-chemical methods have as yet not found very wide application in wine analysis.

Before qualitative or quantitative analysis of the volatile components in a biological material, e.g. wine can be undertaken, such substances must first be isolated. In order that this end may be attained two methods come under consideration:-

A. ISOLATION.

1. Distillation.

For many years a relatively simple steam distillation had been the recognised method of separating the esters. Later distillation under vacuum came into general use. Although the lastmentioned method has the obvious advantage of working at lower temperatures with highly volatile substances, it has the one major drawback that the danger of enzymatic degradation as is nearly always liable to take place in wine, is considerably increased. This is always the case when the distillation is spread over a considerable length of time. Furthermore, when oxidative side-reactions are to be feared it is necessary to undertake the vacuum distillation in a nitrogen atmosphere. With this type of distillation it is imperative that the different distillates are kept at a very low temperature, using dry ice, etc. As a logical development from the above, the so-called Flash Evaporator was developed by White⁴⁸⁾ as based on a suggestion by Milleville and Eskew⁴⁹⁾. White used this apparatus for his work on the volatile components of apples.

This.....

This apparatus is very complicated and requires much expert knowledge and attention.

2. Extraction.

The liquid to be examined is extracted with the solvent which proves to be best-suited to the prevailing conditions.

(a) Ether.

Bayer et al⁴²⁻⁴⁶⁾ used peroxide-free diethyl ether as solvent. This compound is very volatile and dissolves the esters as well as alcohols, aldehydes and ketones in addition to many polyphenolic substances.

(b) Pentane.

According to Mosca and Waser³⁴⁾ and Hennig and Villforth^{35 & 36)} this solvent may be regarded as a "selective solvent" for esters. The low-molecular alcohols and the aromatic aldehydes show very little or no affinity for pentane. The pentane is less volatile than ether. Because of its relatively low boiling point of 34°C, it can easily be quantitatively removed from the rest of the sample. The pentane supplied by the firm E. Merck, Darmstadt, possessed according to our tests, no substances interfering with the ester determination.

B. BASIS OF THE DIFFERENT METHODS OF ANALYSIS FOR ESTERS.

The analytical methods of ester determination are based on the following reactions:-

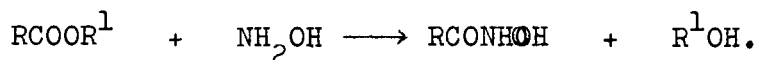
I. Fatty Acid Fraction.

1. Hydrolysis.

In aqueous solution an ester is slowly decomposed into its alcohol and acid components. This reaction is reversible and very slow, but can be catalysed by acids and alkalies, the process being carried to complete degradation by the latter. The identification of an ester consists of the separation of the ester into its component alcohol and acid. Thus it becomes clear why it still remains practically impossible to separate a mixture of esters quantitatively into all its individual components.

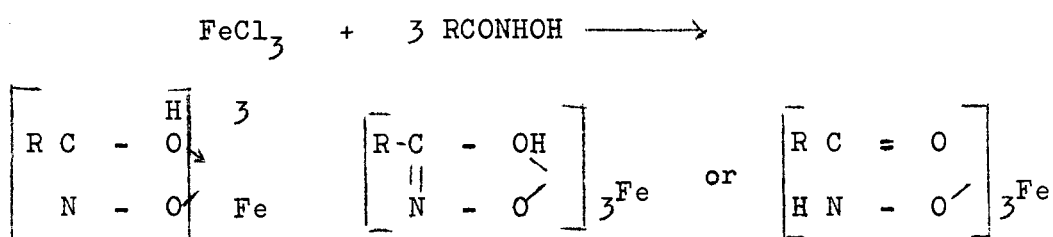
2. The formation of Hydroxamic Acids.

An ester is changed at room temperature by hydroxylamine in the presence of alkali to the corresponding hydroxamic acid.

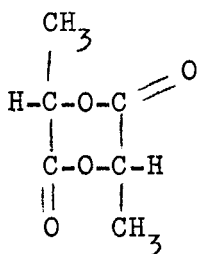


This reaction forms the basis of the qualitative test for carbonic acid ester as shown by Feigl^{52 & 53)} in a concentration range of 2 - 12 micrograms. The hydroxamic acids form strongly coloured red-violet complex compounds when weakly acid ferric chloride solution is added.

Sidgwick⁵⁵⁾ contends that this complex has the following structure:-



Davidson⁵⁶⁾ employed this reaction for the classification of many organic compounds including the esters. The phenols undergo a yellow coloration. The hydroxamic acid test is negative in the case of the carbonates and esters of the inorganic acids. Simple as well as very complicated organic acid esters are positively identified with this reagent. Here special mention must be made of lactic acid. This compound forms an internal ester or lactone which also reacts positively to the hydroxamic acid test.



A phenolic group either in the original compound or in the hydroxylaminolysis of the COOH group does not impair the detection with ferric chloride. Aldehydes and ketones also react positively, thus sufficient reagent should be present.

Bayer⁵⁷⁾ indicated that the complex formation is governed by the amount of ferric chloride and hydroxamic acids

present.....

present and by the acidity of the solution. As these amounts are varied the hydroxamic acid Iron III Complex varies in constitution and colour.

3. Separation as Anions.

Brown⁵⁸⁾ spotted a mixture of the sodium salts of the low-molecular saturated fatty acids onto Whatman No. 1 paper and developed the chromatogram with n-butanol, ethyl alcohol and ammonia. The separated anions, i.e. formate, acetate, propionate, n-butyrate, isobutyrate, n-valerate, isovalerate, n-caproate, isocaproate and n-octanate are fixed and coloured by spraying with a 0,04% aqueous solution of bromothymolblue, pH 7.5 (corrected with NaOH solution). After repeated runs good Rf-values were obtained. The writer could identify salt concentrations down to 5 millimicrons.

Nijkamp⁵⁹⁾ separated the C₁ - C₁₀ straight-chain fatty acids in minimum concentrations of 0.5 mg. per individual substance on a silica gel column impregnated with iso-octane in 95% methyl alcohol and small concentrations of bromothymolblue and ammonia as mobile phase. The separated acids were determined to within an error margin of 5%.

Frey and Wegener⁶⁰⁾ hydrolysed a sample containing 1 - 2 mg. of free and esterified acids. Subsequently the acids were removed by distillation and neutralised with an equivalent quantity of sulphuric acid and then very slightly acidified. The free acids are ether-extracted, the ether removed and dissolved in alcohol. By the round filter technique on Schleicher and Schüll paper 2045a buff, the acids are chromatographically separated. A test mixture is also spotted onto the chromatogram. As developing solution n-butanol : NH₃ : water (80:6:14) was used. After drying the chromatograms were sprayed with 0.04% bromothymolblue solution.

Koster and Slavik⁶¹⁾ were the first to employ acetylated paper for the separation of hydrophobic substances. The non-aqueous solution acts as stationary phase on the paper fibres, whilst the hydrophylic mixture travels along the paper. By acetylating the paper without destroying the fibre structure it can acquire a very strong hydrophobic character.

Micheel and Scheppe^{62, 63, 64)} also employed acetylated paper. They acetylated Schleicher and Schüll 602h

paper according to the D.R.-Patent 184, 201, the dried paper showing an acetyl content of 24 - 25%. This paper is characterised by its resistance to water and excellent absorbent properties. On the paper the saturated straight-chain $C_5 - C_{18}$ fatty acids were separated as hydroxamates. The fatty acids are first changed into the methyl esters by treatment with dioxan. Subsequently equi-molecular quantities of hydroxylamine and sodium hydroxide are added and boiled for two minutes on a waterbath. After the mixture has been neutralised with glacial acetic acid dissolved in tetrahydrofurfurane, a sample is chromatographically separated on the previously described paper. As developing solution acetic acid ethyl ester : tetrahydrofurfurane : water (0.6 : 3.5 : 4.7 volumes) is used.

Struck⁶⁵⁾ used the hydroxamic acid method for the separation of the $C_1 - C_{10}$ aliphatic aldehydes.

Thompson⁶⁶⁾ studied the factors which could influence the hydroxamic acid method. She found that the reaction is best carried out in peroxide-free diethyl ether. Up to 1.3% of water in the reaction mixture in no way impairs the process. Under optimal conditions this reaction reaches its equilibrium when the reaction mixture is heated for 25 minutes at $30^{\circ}C$, at which stage 85% of the straight-chain fatty acids are changed to hydroxamic acids. The absorption spectra of the different hydroxamic acids have their maximum at 520 millimicrons. The optical density of the hydroxamate solution at 520 millimicrons is in direct relation to the ester concentration.

Bayer et al⁵⁷⁾ in accordance with the findings of Thompson dissolved the esters in diethyl ether, converted them into hydroxamic acids and separated them as hydroxamic acid Iron III complexes by adopting a technique whereby the paper is previously impregnated with ferric chloride and dried. By photometric measurements of the extracted violet compounds and comparison with a standard curve, the aliphatic acid esters could be determined in micro-quantities. Upon discussion of the theory of this reaction it was shown that it is absolutely imperative that the experimental conditions be carefully controlled when this method of analysis is applied.

Keulemans and Bayer⁶⁸⁾ and Bayer⁶⁹⁾ suggested a simplified apparatus for the determination of the volatile substances by gas-chromatography. The separating column with the

chamber.....

chamber used for the registration of the heat conductivity are built into an air thermostat. Ethereal oils with boiling points of up to 300°C (Hg 760 m.m.) are separated on different stationary phases.

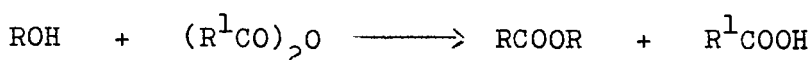
Bergman and Segal⁷⁰⁾ separated the hydroxamic acids two-dimensionally with 95% ethyl alcohol plus 5% ammonia, pyridine and water (3:1:1 volumes) as the first and 95% ethyl alcohol : dioxan : water : acetic acid (60:20:19:1) as the second developing solution. They applied the ferric-chloride spray only as a means of localisation. Their quantitative method was based on the fact that hydroxylamine and hydroxamic acids as shown by Blom⁷¹⁾ are oxidised by iodine in acid medium to nitrous acid. This oxidation is carried out in an acetic acid medium in the presence of a diazotisable aromatic amine (sulphanilic acid). The excess iodine is removed with sodium-thiosulphate and the formed diazo compound is converted into a dark-red azo dye by the addition of *a*-naphthylamine. This red compound is estimated colorimetrically.

II. Alcohol Fraction.

Meigh⁷²⁾ summarised as follows the different reactions which may serve as basis for the micro-determination of the free alcohols.

1. Esterification.

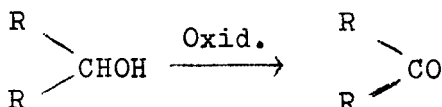
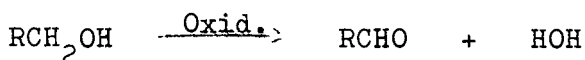
The alcohols combine with acid anhydrides and acid chlorides to form esters.



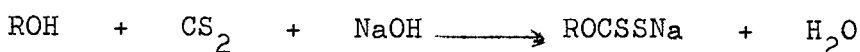
These reactions could be employed for the quantitative micro-estimation.

2. Oxidation.

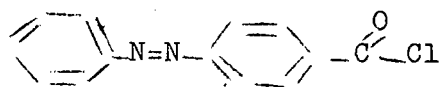
Primary alcohols can be oxidised to aldehydes and further to carbonic acids. Secondary alcohols are more slowly oxidised to ketones.



3. Xantogenate.....

3. Xantogenate formation.

Woolfolk et al⁷³⁾ used p-Phenylazobenzoylchloride,



for the chromatographic separation of the alcohols. Although this compound shows excellent possibilities for the separation of macro-chemical quantities of the lower alcohols it proved less successful for micro-chemical separation.

Frey and Wegener⁷⁴⁾ hydrolysed a sample containing free and esterified alcohols for one hour under reflux. The higher alcohols were extracted with carbon tetrachloride and in this solvent they were oxidised for eight hours after the addition of potassium bichromate sulphuric acid, neutralised and acidified with sulphuric acid. The formed fatty acids were extracted with ether and chromatographed for three hours on a round filter chromatogram. Upon spraying with colour reagent valeric, butyric and acetic acids were found, corresponding to amyl, butyl and ethyl alcohols.

Riley⁷⁵⁾ used 2,4-dinitrophenylpropionyl chloride as ester forming reagent. The esters were subsequently colorimetrically estimated at 242 millimicrons.

Reichstein⁷⁶⁾ was the first to use 3,5-dinitrobenzoyl chloride for macro-chemical determination of alcohols.

Rice et al⁷⁷⁾ and Meigh^{78 & 79)} also applied this compound for the quantitative paperchromatographic determination of the alcohols. Meigh added to a petroleum-ether solution of the alcohols a few drops of pyridine and a solution of 3,5-dinitrobenzoyl chloride dissolved in benzene. The solution was boiled under reflux. Afterwards the pyridine was removed by repeated washings with dilute hydrochloric acid, which in its turn was removed by washing with dilute sodium hydroxide. The latter was removed by repeated small washings of distilled water. The solution containing the dinitrobenzoates was concentrated and chromatographed. As developing solution heptane saturated with methyl alcohol was used in a chamber saturated with the abovementioned solution as well as with its inverted phase. The chromatograms were dried at room temperature. After spraying with a 0,002% solution of

Rhodamine in methyl alcohol and subsequent drying, the 3,5-dinitrobenzoic acid esters were readily discernible under ultra violet light. For the quantitative estimation the spots are not sprayed beforehand but are rendered discernible under ultra violet light by the following expedient. A clean sheet of chromatographic paper is sprayed with Rhodamine solution and dried. This sheet is used as fluorescent background for the chromatogram proper. The spots are demarcated and cut out, cut into small strips and extracted with methyl alcohol. The latter is quantitatively evaporated, cyclohexane added and the absorption determined at 242 millimicrons. Although it was not possible to apply this method for the quantitative determination of the alcohols during the course of this study, the qualitative identification of the alcohols by this method proved most satisfactory.

Kariyone et al⁸⁰⁾ used the xantogenate formation as basis for the alcohol estimation and used as developing solution 2% potassium hydroxide in n-butyl alcohol.

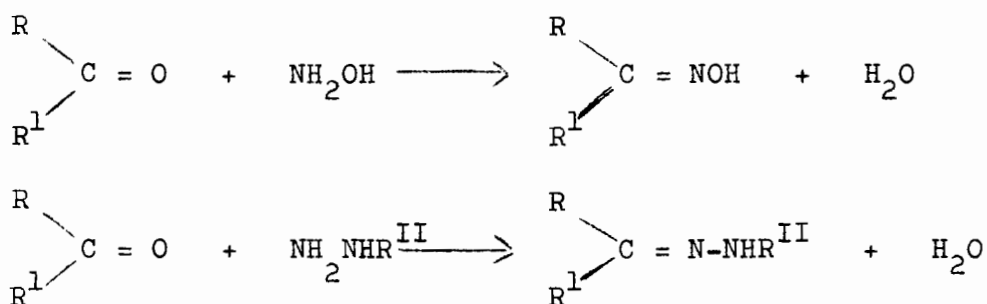
A definite improvement was attained by Pohloudek-Fabini and Beyrich⁸¹⁾ when they separated the xantogenates on phosphate buffered (pH 12) paper with water-saturated butanol as mobile phase. They identified the spots after chromatography either by viewing under ultra violet light or by spraying with ammoniummolybdate-sulphuric acid, coppersulphate or nickel sulphate solutions.

C. BASIS OF THE DIFFERENT METHODS OF ANALYSES FOR ALDEHYDES AND KETONES.

Meigh⁸²⁾ grouped the different reactions which could serve as basis for the micro-chemical determination of the aldehydes and ketones as follows:-

1. Condensation.

Aldehydes and ketones combine with hydroxylamine to form oximes and with substituted hydrazines, hydrozones are formed.

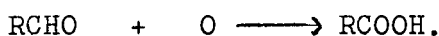


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A large number of the hydrazones can be separated by chromatography.

2. Oxidation.

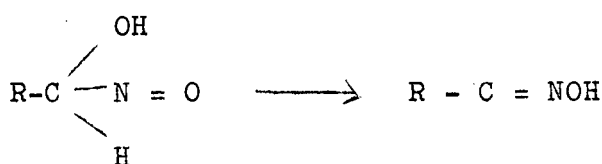
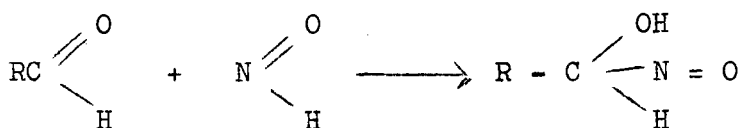
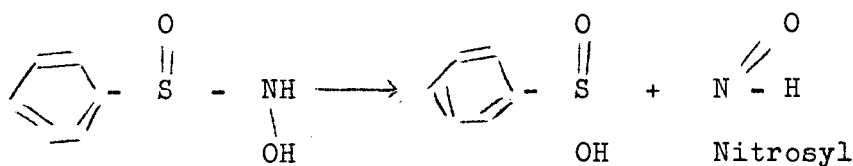
Aldehydes and especially saturated aliphatic aldehydes are readily oxidisable to the corresponding fatty acids.



The ketones are not as readily subjected to this oxidation and they form more than one type of product.

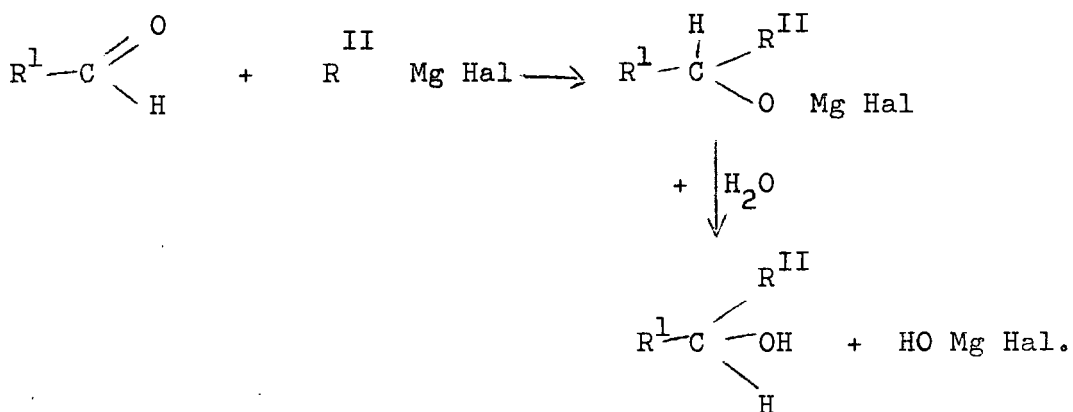
3. Reaction with Bensulphhydroxamic acid.

The transformation of the aldehydes with bensulphhydroxamic acid according to Struck⁸³⁾, i.e. the application of the reaction of Angeli Rimini according to Gattermann⁸⁴⁾.



4. Separation as Grignard Derivatives.

Seligman et al⁸⁵⁾ used the Grignard derivatives to separate aldehydes.



Rice et al⁸⁶⁾ and Meigh⁸⁷⁾ separated the 2,4-dinitrophenylhydrazones of different aldehydes and ketones and attained the following results. Rice et al used as mobile phase 5% ether in petroleum ether of boiling point range 65 - 110°C, the chromatograms being developed upwards. Meigh employed methyl alcohol : heptane, the chromatograms being developed by downward irrigation to a distance of 20 cm. at + - 20°C.

TABLE XV. Rf-VALUES OBTAINED FOR DIFFERENT ALDEHYDES AND KETONES BY RICE et al and MEIGH.

Aldehydes and Ketones	Rf-value Rice et al	Rf-value Meigh
Formaldehyde	0.75	0.09
Acetaldehyde	0.79	0.18
Propionaldehyde	0.57	0.32
Acetone	0.85	0.30
n-Butylaldehyde	0.85	0.42
Methyl-ethyl ketone	0.90	0.43
n-Valeraldehyde	-	0.54
Methyl-propyl ketone	0.90	0.53
Crotonaldehyde	-	0.22
2-Hexanal	0.82	0.52
Furfural	0.57	0.06
Glyoxal	-	-
Diacetyl		
Pyruvic acid	-	
Benzaldehyde	0.68	-
Salicyl aldehyde	0.30	-
Cinnamic aldehyde	0.48	-
2,4-Dinitrophenylhydrazine	0.05	

Schulter and Storp⁸⁸⁾ mentioned that the aldehydes are very strongly inclined towards autoxidation and that this property rapidly increases in the aliphatic range as the vapour pressure rises and the carbon atom number decreases. This limits the application of paper chromatography for the separation of aldehydes.

The phenylhydrazones on the other hand exhibit a small vapour pressure. Losses through oxidation or evaporation

are.....

are negligible to the extent Ethylenediamine hydrate can be detected as 2,4-dinitrophenylhydrazone. The abovementioned authors separated as 2,4-dinitrophenylhydrazones the $C_8 - C_{14}$ aldehydes on Schleicher and Schüll 2043 paper with methyl alcohol : acetic acid (9.25 : 0.75) as mobile phase. By spraying with 10% potassium hydroxide solution the 2,4-dinitrophenylhydrazones are rendered more discernible. As a further reagent for the identification a 0.5% solution of benzidine in acetic acid : ethyl alcohol (4:1) is used as a spray before viewing under ultra violet light.

Jegerow and Borissowa⁸⁹⁾ used the 2,4-dinitrophenylhydrazones as a basis for the quantitative determination of the keto acids in wine. Joppien⁹⁰⁾ found in the course of his investigations on thujone compounds that the 2,4-dinitrophenylhydrazones of the two isomers of thujone show excellent mobility in benzidinehydrocarbons and that their solubility in pentane was good. In a range of experiments with different aldehydes and ketones it could be proved that the dinitrophenylhydrazones of the benzene derivatives show practically no mobility in petroleum ethers. The dinitrophenylhydrazones of the saturated aliphatic aldehydes and ketones increase in their solubility, i.e. their mobility as the length of the chain increases, whilst the terpene derivatives as far as they were included by these authors in their experiments had their mobility, although satisfactory, governed by the position and number of double bonds. The maximum absorption for the 2,4-dinitrophenylhydrazones of the aliphatic aldehydes and ketones ranges from 325 - 365 millimicrons.

Salo and Suomalainen⁹¹⁾ used the dinitrophenylhydrazones for the colorimetric determination of diacetyl in spirit. Pure pyridine proved to be an excellent extracting agent for these compounds. Ethylenediaminhydrate was an excellent reagent for colouring the pyridine extract. The violet colour is not very stable and possesses an extinction maximum at 530 millimicrons.

Gordon et al⁹²⁾ used the silicic acid gel as supplied by E. Merck, Darmstadt, mixed with celite analytical filter aid ex John Manville Co. for the separation and determination of the dinitrophenylhydrazones of the saturated $C_1 - C_4$ aldehydes. They found these yellow compounds to have their absorption maximum at 354 millimicrons.

Mukherjee⁹³⁾ estimated minute quantities of iron in the following manner. The Schiff's base resulting from the action of salicylaldehyde on glycinehydroxamic acid is transformed into a violet compound in the presence of small amounts of iron.

Struck⁹⁴⁾ transformed the aldehydes by subjecting them to the reaction with bensulph-hydroxamic acid to the corresponding hydroxamic acid. The mixture is chromatographed on Whatman No. 1 paper with butanol : acetic acid : water (4:1:5) as mobile phase. Subsequently the aldehyde hydroxamic acid esters are localised by spraying with ferric chloride. The benzenesulphenic acid resulting from the described reaction as well as the unchanged bensulph-hydroxamic acid could be distinguished by their reddish-yellow discoloration of the ferric chloride. Formaldehyde alone can also be chromatographed as hexamethylenetetramine in very minute concentrations.

Bayer et al⁹⁵⁾ and Bayer⁹⁶⁾ contended that their method for the separation of the fatty acids adapted to the aldehydes is more advantageous. With bensulph-hydroxamic acid the aldehydes were changed into the corresponding hydroxamic acids, the latter being separated as hydroxamic acid Iron III complexes on paper which had previously been impregnated with ferric chloride. Concentrations corresponding to 10 micrograms of the $C_1 - C_{10}$ aldehydes are still detectable. The ketones and the aromatic aldehydes, i.e. salicylaldehyde, vanilline, etc. are not transformed to hydroxamic acids.

Newcombe⁹⁷⁾ separated the aldehydes and ketones by employing their differing reaction rate under different conditions. The chromatograph paper was previously sprayed with different reagents, i.e. sodium hydrosulphite, sodium carbonate and sec. di-sodium phosphate. Aldehydes are more strongly hampered in their mobility than the ketones. Vanilline remains on the starting point when the paper is treated with sodium carbonate, while other aldehydes present move from the starting point.

Haeseler and Vogl⁹⁸⁾ indirectly determined the acetal content by first colorimetrically determining the total aldehyde in acidic medium with fuchsine-sulphurous acid, then afterwards determining the free aldehyde in basic medium with sodium-nitroprusside.

The work of Seligman et al⁹⁹⁾ referring to separation by means of the Grignard derivatives has already been cited.

Deckenbrock¹⁰⁰⁾ chromatographed a concentrated ether extract in butanol : acetic acid : water (4:1:5) for the separation of vanilline and ethylvanilline. As cinnamic aldehyde is insoluble in ether it was extracted with pentane and likewise chromatographed. The different spots could be identified by spraying with hydrazine sulphate in hydrochloric acid and viewing under ultra violet light.

MICRO-DETERMINATION OF THE ESTERS.

A. Determination of Fatty Acids.

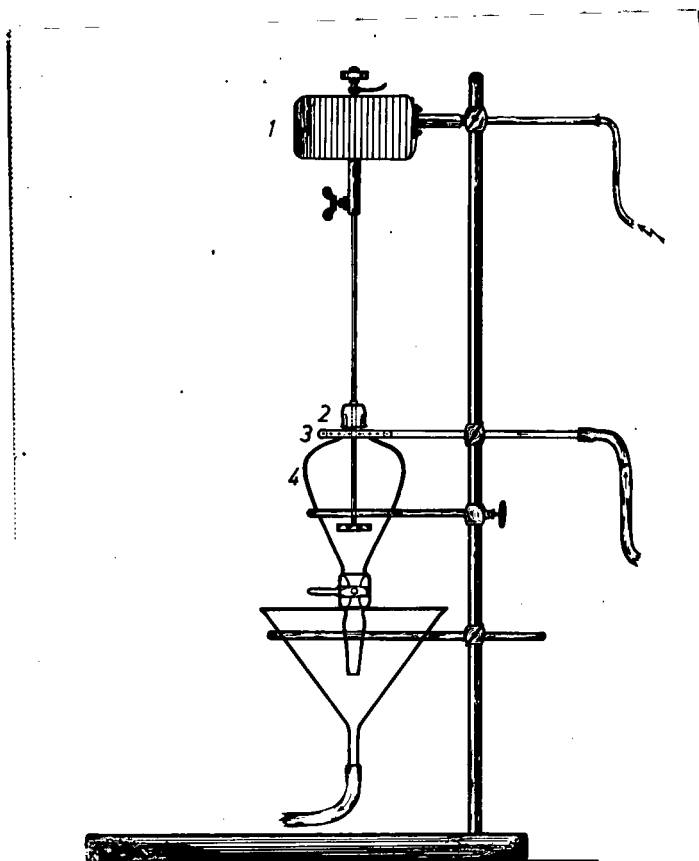
Reagents.

1. Pentane (for the chromatography) E. Merck, Darmstadt.
2. Carbon tetrachloride, E. Merck, Darmstadt.
3. Sodium sulphate (anhydrous).
4. Absolute alcohol.
5. Hydroxylamine reagent.
 - (a) 5% Hydroxylamine hydrochloride dissolved in methanol. This solution is freshly prepared every second day.
 - (b) 12.5% Hydroxide in methyl alcohol.
 - (c) Equal volumes of (a) and (b) are pipetted together, shaken and filtered. This solution is to be freshly prepared every four hours (Compare Coddu et al¹⁰¹⁾).
6. 32% Hydrochloric acid.
7. 2% Ferric chloride (anhydrous) in methyl alcohol to which 1% Hydrochloric acid is added.
8. Developing solutions:-
 - (1) Dimethylformamide : n-butyl alcohol : water (5:45:50).
 - (2) Acetic acid ester of ethyl alcohol : tetrahydrofurane : water (0.6 : 3.5 : 4.7).
9. Schleicher and Schüll paper: 2043 mg1. or 2043b acetylated.
10. 5% Sodium acetate.
11. 1.3% Iodine in glacial acetic acid.
12. 1% Sulphanilic acid in 25% glacial acetic acid (V/V).
13. 0.1% Sodium-....

13. 0.1% Sodiumthiosulphate solution.
14. 0.6% Napthylamine in 30% glacial acetic acid (V/V).

Five hundred ml. wine is consecutively extracted with 150, 100 and 50 ml. of pentane, i.e. 300 ml. of pentane in all. The 500 ml. wine is measured into a thick-walled separating funnel, the pentane added and the contents of the funnel agitated for five minutes with the aid of a vibrator mixer as supplied by Bopp and Reuther, GmbH, Mannheim. The resulting emulsion is allowed to settle for fifteen minutes. A few drops of absolute alcohol generally suffices to separate the emulsion into two phases.

Diagram III.

Extraction Apparatus.Description:

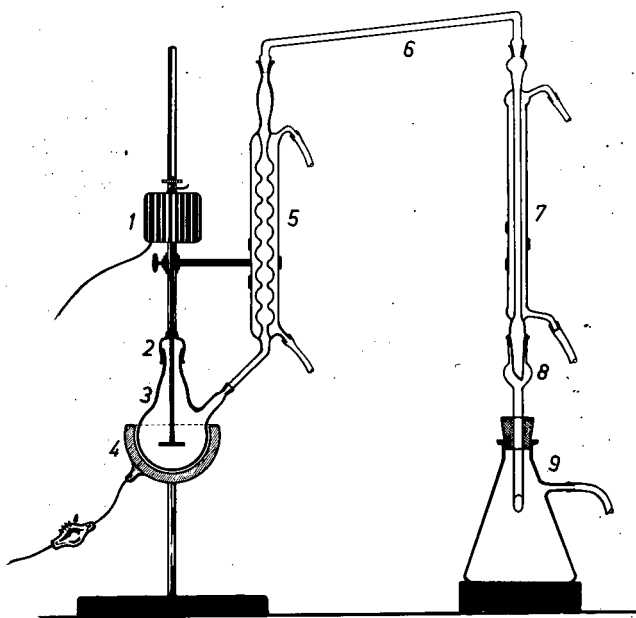
Into a separating funnel (4) of one litre capacity the rod of the Vibro Mixer (1) (as supplied by the firm Bopp and Reuther, Mannheim) with a mixing flange of 2 cm. in diameter is passed. The funnel acts as receiver for the wine and solvent, i.e pentane or ether. The funnel itself is sealed with a perforated rubber cap (2). For the water-cooling of the mixture during the agitation a finely perforated ring-shaped copper tube is passed round the shoulder of the funnel. The cooler water is received in a large funnel and drained away.

The intensity of the agitation can be controlled by manipulating a lever on the vibrator mixer for this purpose.

After.....

After every addition of pentane the solvent must be separated from the wine in the separating funnel. The pentane extracts are combined in a 300 ml. measuring flask. After the third portion of pentane has been added the flask is filled up to the mark with pentane. The contents of the flask must be well mixed. From this 300 ml., 150 ml. is pipetted out for hydrolysis and subsequent alcohol estimation. The fatty acids are determined in the remaining 150 ml. To this volume 3-4 gm. of anhydrous sodium sulphate is added, shaken and left to dry overnight in a refrigerator. This latter mixture is then filtered through a glass-wool filter into a 250 ml. Jena glass distilling flask with S.29 ground joint and side tube S.14.5. The residue is washed three times with 5 ml. portions of carbon tetrachloride which is added to the pentane. Subsequently the pentane is very carefully distilled off, the heating jacket being adjusted to the second stage.

Diagram IV.

Apparatus for the Removal
of Volatile Solvent.Description:

The basic apparatus remains the same up to (5) (i.e. the same as the apparatus for hydrolysis, see Diagram V).

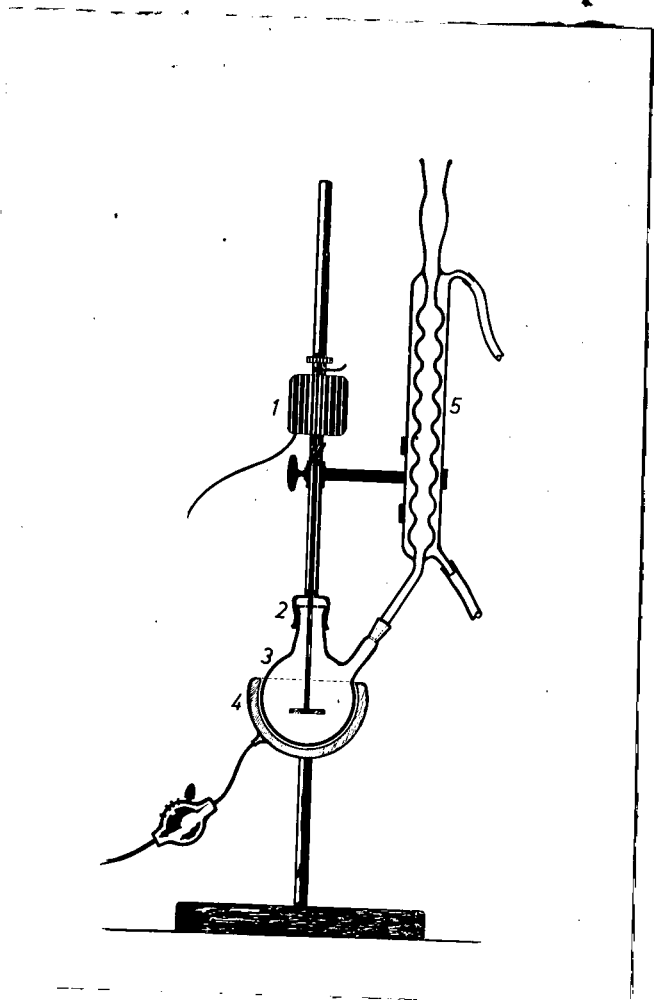
This basic apparatus is joined to the apparatus here described by means of the joining tube (6) to a second condenser (7) which is again joined by means of funnel (8) to a suction flask (9).

Before distillation, the flow of water to condenser (5) (see Diagram V) is turned off while the cooler (7) is strongly supplied with cooling water for the condensing of the solvent to be removed by distillation.

By means of the heating jacket (4) the temperature can be regulated.

Diagram V.....

Diagram V.

Apparatus for Hydrolysis.Description:

The rod of the vibrator mixer (1) is passed into a 250 ml. double-necked distilling flask. This flask is heated by means of the heating jacket (4), the temperature of which can also be regulated. The flask is sealed by means of a perforated rubber cap (2). Attached to the side-tube is a condenser (5) acting as reflux condenser. The simultaneous mixing and heating of liquids can be undertaken in this apparatus.

After.....

After sufficient time has been allowed for the carbon tetrachloride to cool, the rubber cap (2) is removed and 1 ml. of the hydroxylamine reagent is added and the cap replaced. The heating jacket is adjusted to the first stage, the vibrator mixer set in motion and the mixture allowed to react under reflux for fifteen minutes and then allowed to cool. The mixture is quantitatively washed with carbon tetrachloride from the distilling flask into a 25 ml. measuring flask. After filling up to the mark with carbon tetrachloride the mixture is neutralised with 0.115 ml. of 32% HCl. Subsequently the flask is shaken uninterruptedly for five minutes.

Two phases separate in the measuring flask. On the carbon tetrachloride a layer is formed in which the hydroxylamine reagent and the hydroxamic acid esters are found. From the middle of this layer the sample for spotting onto the paper is later removed by means of an Agla micro-pipette. Separation, filtration and concentration are superfluous.

Two hours are allowed to elapse before 0.01 to 0.1 ml., depending upon the concentration of the esters in the original mixture, is spotted onto the paper.

With dimethylformamide : n-butyl alcohol : water as mobile phase the hydroxamic acids are chromatographed by downward irrigation for sixteen hours at $20^{\circ} \pm 1^{\circ}\text{C}$. A test mixture is spotted next to the unknown mixture. After the paper is dried, ferric chloride solution is used for the localisation of the different hydroxamic acids, blue-violet hydroxamic acid Iron III complexes being formed. For comparison the same paper previously bathed with the ferric chloride solution and dried can be used for the chromatography, i.e. the iron complexes rather than the hydroxamic acids being separated.

The average R_f-values, i.e. the average value obtained for ten runs, five times singly and five times in a different mixture of at least four different hydroxamic acids, are presented in the following table:-

TABLE XVI. THE R_f-values FOR DIFFERENT HYDROXAMIC ACIDS AND HYDROXAMIC ACID IRON III COMPLEXES.

Ester of	R _f -value Hydroxamic Acids	R _f -value of the Hydroxamic acid Iron III Complexes
Formic acid	0.55	0.23
Acetic acid	0.64	0.33
Propionic acid	0.77	0.54
Butyric (isobutyric) acid	0.83	0.66
Isovaleric (valeric) acid	0.90	0.80
Caproic acid	0.92	0.88
Capric acid	0.95	0.96
Cinnamic acid	0.90	0.90
Salicylic acid	0.82	0.82
Anthranilic acid	0.96	0.96

These values compare well with values obtained by Bayer et al. Both these methods of separation have the apparent disadvantage that the homologues starting from C₆ upwards, together with the aromatic acids, remain cluttered together. This impedes the quantitative determination of such compounds. Furthermore, the tendency towards smudging seems to be more pronounced in the case of the ferric chloride treated paper. Bayer et al extracted the blue-violet complexes with methyl alcohol and measured the extinction of the resulting solution at 530 millimicrons. When the uni-dimensional chromatography of the hydroxamic acids indicate the presence of higher homologues and aromatic compounds then a two-dimensional chromatography developed in the course of this study is applied with dimethylformamide : n-butyl alcohol : water as the first and acetic acetate : tetrahydrofurfurane : water as the second developing phase. The spotting onto the paper is undertaken in the following manner. A sheet of acetylated Schleicher and Schüll 2043b paper of the dimensions 40 x 60 cm. is used. Ten cm. from the one narrow border the samples are spotted onto the paper and 6 cm. from the right-hand side of the paper a test mixture containing the hydroxamic acids of C₂, C₄ and C₅ fatty acid esters is spotted. The sample to be separated is spotted

in equal volumes 12 cm. and 18 cm. from the righthand side. After the spots have been allowed to dry the chromatogram is developed downwards with dimethylformamide : n-butanol : water (0.5 : 4.5 : 5.0) for sixteen hours.

After drying a strip 14 cm. wide is cut away from the righthand side of the paper. This strip is sprayed with ferric chloride solution. From this strip the position of the C_5 acid in the test mixture can be registered. Furthermore, it should be possible to see that in the sample to be examined the acids from C_5 upwards, as well as the aromatic and fatty acids are only partially separated. The remaining strip of paper which remains unsprayed is cut off as close as possible above the C_5 spot. This paper is turned through 90° to the original direction and developed upwards in ethyl acetate : tetrahydrofurfurane : water (0.6 : 3.5 : 4.5). Before developing, another test mixture containing the hydroxamic ester of C_5 , C_8 , C_{10} and an aromatic ester, is spotted onto this paper about 8 cm. from the righthand side and on the same line on which the unsprayed original mixture was spotted. Next to this test mixture the original mixture to be examined is once more spotted. After ten hours irrigation the chromatogram is dried and again the righthand portion of the paper is cut off and sprayed with ferric chloride. Now it can be observed that the hydroxamic acids unseparated by the first run have separated themselves. From the Rf-values the different compounds can be identified.

Apart from the fact that according to this method a better separation is attained, cinnamic and salicylic acids (as hydroxamic acids) show Rf-values of 0.45 and 0.5 respectively in the first run, rendering them immediately recognisable.

Diagram VI. Procedure for Two-Dimensional Development of Chromatogram for Lower Saturated and certain Aromatic Fatty Acids.

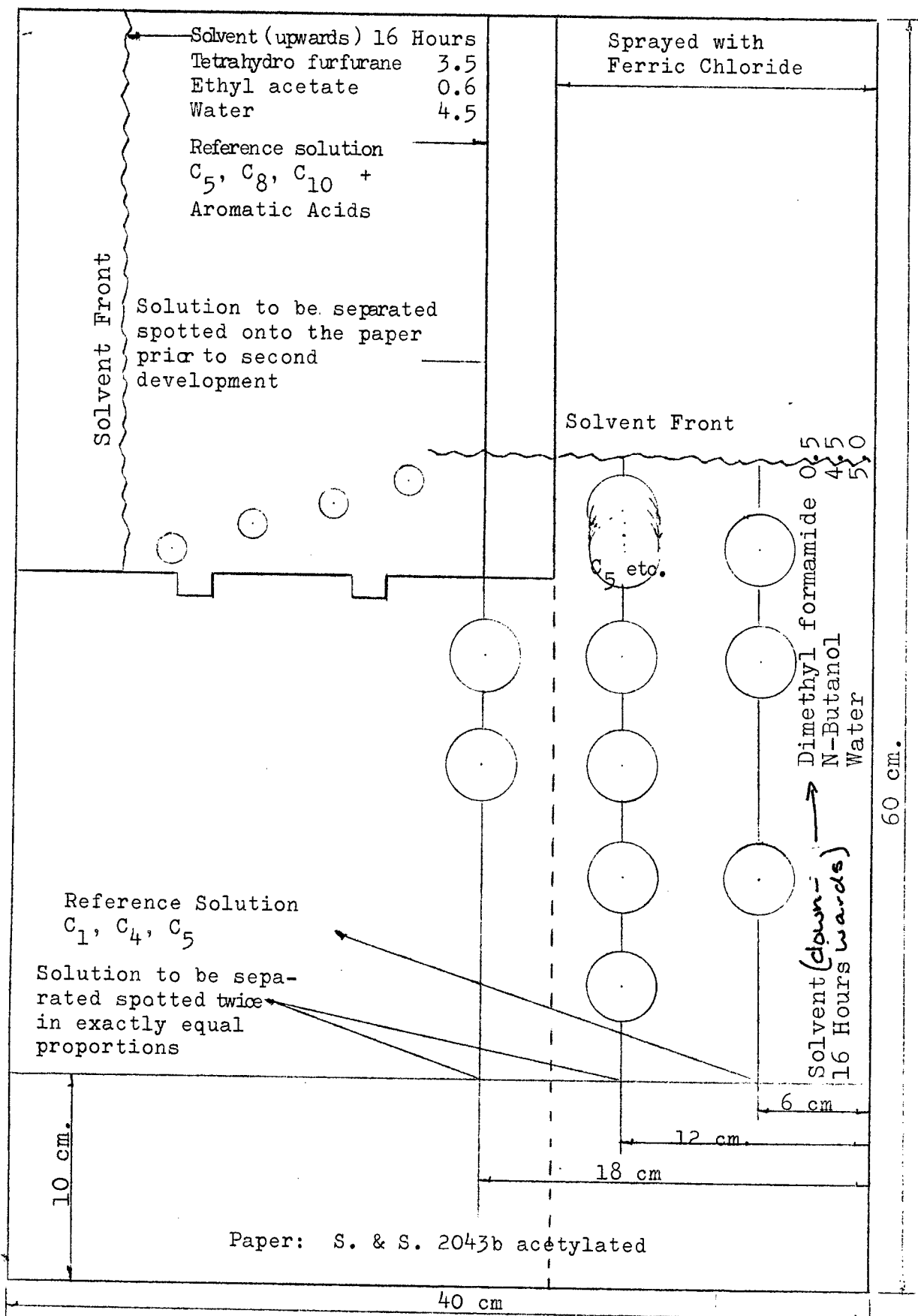


TABLE XVII. THE R_f-VALUES FOR DIFFERENT HYDROXAMIC ACIDS
IN DIMETHYLFORMAMIDE : n-BUTYL ALCOHOL : WATER
(5:45:50) DEVELOPED DOWNWARDS FOR SIXTEEN
HOURS AT 20° ± 1°C ON SCHLEICHER AND SCHÜLL
2043b ACETYLATED PAPER.

Hydroxamic Acid of	R _f -Value
Phthalic acid	0.18
Formic acid	0.30
Acetic acid	0.40
Propionic acid	0.54
Butyric acid	0.65
Isovaleric acid	0.70
Caproic acid	0.77
Cinnamic acid	0.45
Salicylic acid	0.56
Benzoic acid	0.60
Anthranilic acid	0.69

TABLE XVIII. THE R_f-VALUES FOR DIFFERENT HYDROXAMIC ACIDS
IN ETHYL ACETATE (0.6 : 3.5 : 4.7)
DEVELOPED UPWARDS FOR TEN HOURS AT 20° ± 1°C
ON SCHLEICHER AND SCHÜLL 2043b ACETYLATED PAPER.

Hydroxamic acid of	R _f -Value
Isovaleric acid	0.84
Caproic acid.	0.79
E nanthic acid	0.75
Caprylic acid	0.70
Pelargonic acid	0.58
Capric acid	0.53
Laurine acid	0.36
Anthranilic acid	0.69

All the R_f-values in the above two tables are the average values for ten runs, each individual being chromatographed five times singly and five times in a mixture containing at least four of the abovementioned compounds.

The Quantitative Determination.

The quantitative determination is carried out according to the method suggested by Bergman and Segal⁷⁰⁾ spraying with ferric chloride, which is applied for the purpose of localisation only. From the chromatogram a piece approximately 2 x 2 cm. containing the hydroxamic acid is cut. This piece of paper is cut into small strips and placed in a 10 ml. measuring flask. The strips are extracted with 5 ml. of sodium acetate under intermittent shaking for fifteen minutes. Then 0.5 ml. of the sulphanic acid and five drops of iodine solution are added and the mixture once more thoroughly shaken. After waiting for five minutes the excess iodine is removed by shaking with two to three drops of sodium thiosulphate solution. Now three drops of α -naphthylamine solution are added and the resulting reddish solution filled with distilled water to the mark. Once more the contents of the measuring flask is shaken and its extinction determined after ten minutes in an Elko III (Zeiss Oberkochen) photometer, using filter S.53.E. and a 2 cm. cuvette. As a blank a piece of paper is removed from near the edge of the chromatogram (not containing any hydroxamic acid) and treated as discussed above.

Standard Graph.

From a standard solution with an ester content of 1 gm. per 100 ml. (referred to as acetic acid) exactly controlled volumes of ester are pipetted by means of an Agla pipette into a double-necked distilling flask (see previous description) containing 15 ml. of pure carbon tetrachloride. To this ester solution, hydroxylamine solution is added and the same procedure followed as previously described in the description of the analytical methods.

From the methyl alcohol layer exact volumes of 0.01, 0.02, 0.025, 0.03, 0.035, 0.04 and 0.05 are brought onto the chromatogram paper, each volume being spotted twice on adjacent starting points. One spot is used for localisation with ferric chloride, the other for cutting out and quantitative determination as azo dye. The volume of the methanol layer is always regarded as constant at 1.0 ml. i.e. when the 1 mg. of ester calculated as acetic acid was originally added prior to hydrolysis, then 0.01 ml. of the layer contains 0.01 mg. of the substance to be determined. This calculation follows after

addition.....

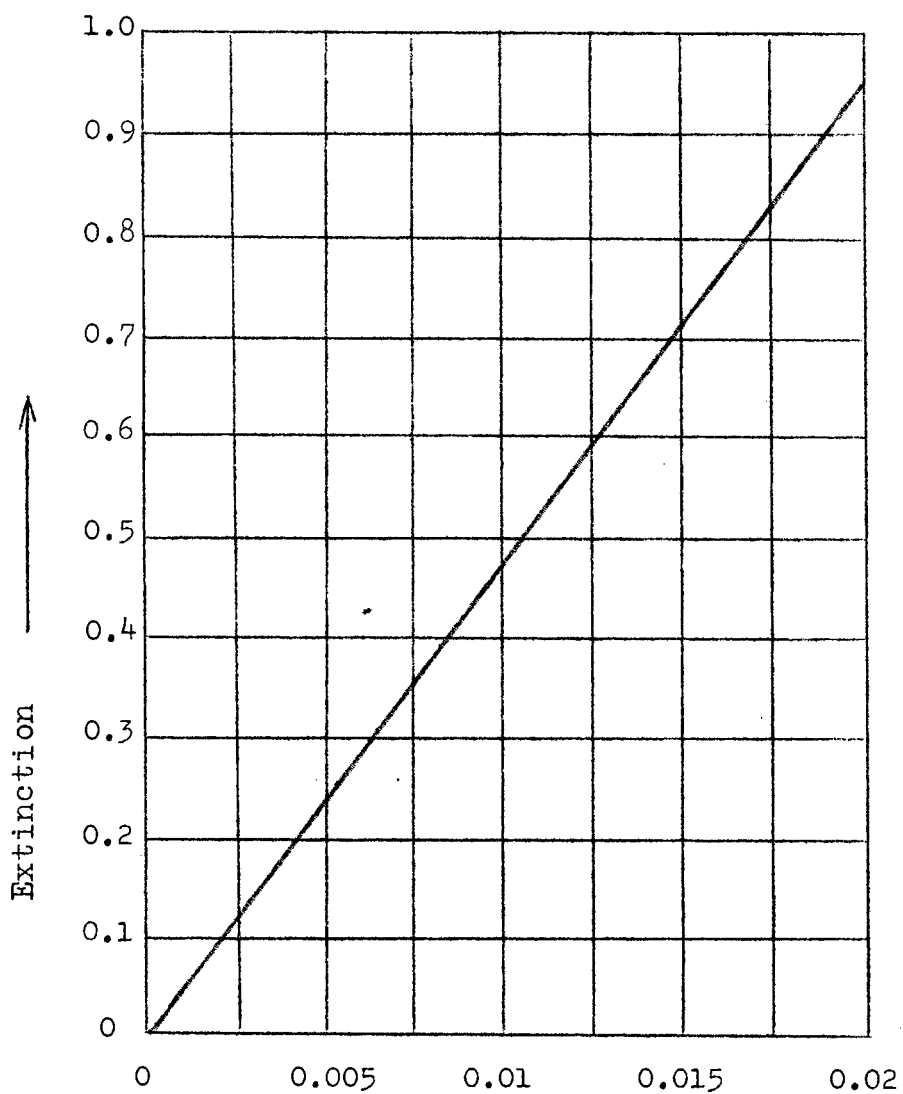
addition of hydroxylamine and neutralisation with hydrochloric acid.

After sixteen hours of downward irrigation with dimethylformamide : butanol : water the paper is cut into strips, the duplicate strip for each spot being sprayed with ferric chloride. From the unsprayed strips of paper the portions corresponding to the violet spots on the treated strips are removed and cut up into smaller strips, extracted, oxidised, coloured and colorimetrically measured as discussed above.

Graph No. 7 represents the relationship between extinction and concentration in an Elko III Filter S.53.E. (2 ml. cuvettes) for all the saturated aliphatic acids examined in the course of this work, i.e. formic, acetic, propionic, butyric, isobutyric, valeric, isovaleric, capric, caproic, enanthic, pelargonic and caprylic acids as well as benzoic acid.

Graph No. 8 shows the extinction in relation to concentration for phthalic, cinnamic, anthranilic and salicylic acids, all calculated as acetic acid.

Graph No. 7. Standard Graph for the Determination of Acetic Acid.



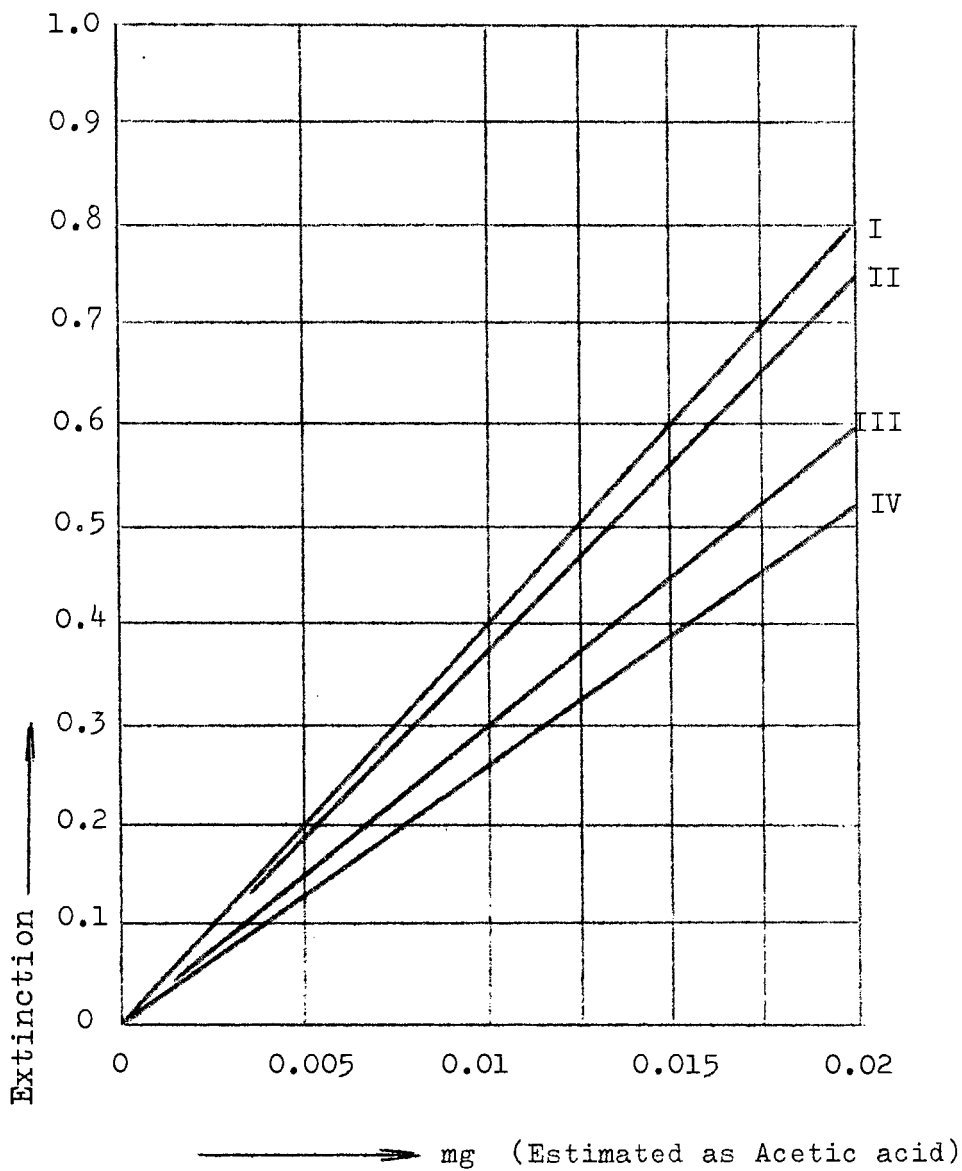
Acetic acid → mg

Filter S.53.E

Cuvette 2 cm.

Graph No. 8. Standard Graph for the Determination of Cinnamic, Salicylic, Anthranilic and Phthalic Acids.

- I. Cinnamic acid.
- II. Salicylic acid.
- III. Anthranilic acid.
- IV. Phthalic acid.



Filter S.53.E

Cuvette 2 cm.

Check on the Method.

A wine sample gave the following values (averages for four analyses).

Fatty Acids (Calculated as ethyl acetate).

	mg/l.
Formic acid	5.6
Acetic acid	48.2
Butyric acid	2.6
Isovaleric acid	10.2
Caproic acid	+

To this wine different quantities of different esters (calculated as ethyl acetate) were added and determined by analysis, the average values for four analyses being furnished.

TABLE XIX. THE AMOUNTS DETERMINED AFTER ADDITION OF DIFFERENT ESTERS TO A WINE.

	Added mg/l.	Found (Average of four mg/l. analyses)
1. Formic acid	10	9.3
n-Butyl ester	25	22.2
2. Formic acid	10	9.6
Ethyl ester	50	48.2
3. Acetic acid	10	9.8
Methyl ester	25	24.4
4. Acetic acid	10	9.6
Iso-amyl ester	50	47.8
5. Butyric acid	10	9.5
Methyl ester	25	23.9
6. Isobutyric acid	10	9.9
Isobutyl ester	50	48.3
7. Isovaleric acid	10	9.8
Ethyl ester	25	24.3
8. Isovaleric acid	10	9.5
n-Amyl ester	50	48.0
9. Anthranilic acid	10	9.5
Methyl ester	25	23.5
10. Salicylic acid	10	9.8
Isobutyl ester	25	22.9
11. Cinnamic acid	10	9.4
Ethyl ester	25	23.7

B. Determination of the Alcohols.

According to Rauen¹⁰²⁾ the rate of hydrolysis K_2 for acetic acid ethyl ester equals 100 and for formic acid ethyl ester 213,000. After an hydrolysis in aqueous solution under reflux (length 120 cm.) lasting twelve hours, 95% of this ester was hydrolysed.

With the vibrator mixer according to Bopp and Reuther it was possible to hydrolyse directly in pentane (see Diagram No. V). After an hour 100 mg. of acetic acid ethyl ester was completely hydrolysed according to the above process whilst 100 mg. of formic acid ethyl ester (calculated as ethyl acetate) was hydrolysed to the extent of 40%. The addition of an emulgator like sodium lauryl sulphate as suggested by Schriener and Fuson¹⁰³⁾ did not prove to be very successful.

The hydrolysis could not be carried out in a diethylene glycol solution as suggested by Redemann and Lucas¹⁰⁴⁾ as this substance because of its strongly positive reaction to most tests for alcohol, impairs the quantitative estimation of the alcohols.

Reagents:

1. Pentane (E. Merck, Darmstadt).
2. 20% Sodium hydroxide.
3. Carbon disulphide.
4. Schleicher and Schüll 2043b mg. paper. The chromatogram is previously cut out and bathed in 1% trisodium phosphate solution (pH 12.0) and dried for ten minutes at 100°C.
5. Mobile phase: water-saturated butanol.
6. Dithio-oxalic acid diamide, 0.5% in 100 ml. absolute alcohol (Riedel de Haen, Seelze, bei Hannover).
7. 10% Nickel sulphate solution.
8. 35% Ammonia.
9. Concentrated hydrochloric acid.

One Hundred and fifty ml. of the pentane extract, as already described, is transferred into a double-necked distilling

flask.....

flask and 5 ml. of 20% sodium hydroxide added and subjected to a hydrolysis under reflux lasting one hour. Subsequently the pentane is very gently distilled off and the residue in the flask heated for another hour on the second stage of the heating jacket. During hydrolysis and subsequent heating the contents of the boiling flask is continuously agitated by means of the vibrator mixer. After cooling 0.4 ml. of carbon disulphide is added. The mixture is well shaken and allowed to stand for twelve hours, then 5 - 10 ml. of pentane is added to dissolve the excess carbon tetrachloride. The pentane is slowly evaporated at approximately 35°C. The xantogenate mixture can be transferred to a dry test tube and kept for two days.

The latter mixture is brought onto the described buffered paper and developed upwards for twelve hours in water-saturated butyl alcohol. Subsequently the paper is dried at 80°C for ten minutes. The xantogenates of the lower saturated aliphatic alcohols are well discernible under ultra violet light, yielding dark spots. Afterwards the chromatogram is sprayed with a 10% solution of nickel sulphate, whereby the xantogenate assumes a deep yellow to reddish colour.

TABLE XX. THE R_f-VALUES FOR XANTOGENATE

Xantogenate of	R _f -Value
Methyl alcohol	0.22
Ethyl alcohol	0.36
Propyl and Isopropyl alcohol	0.56
Butyl and Isobutyl alcohol	0.68
Amyl alcohol	0.80
Hexyl alcohol	0.82

These values were attained as the averages for ten determinations, each substance being chromatographed five times singly and five times in a mixture of at least three of the above substances.

Quantitative.....

Quantitative Determination.

After spraying with nickel sulphate the chromatograms are allowed to dry for ten minutes at room temperature. Subsequently they are placed in a glass trough and washed six times with distilled water, removing the excess nickel sulphate. The nickel complex of the xantogenates is extremely stable. The chromatogram is dried for ten minutes at 100°C.

The spots (excluding the spot for ethyl alcohol) are cut out and incinerated and the ash subjected to a micro-determination for nickel.

The nickel-containing ash after cooling, is dissolved in two drops of hydrochloric acid to which four drops of ammonia solution are subsequently added. Two drops of dithio-oxalic acid diamide are added. The resulting red solution is quantitatively washed into a 10 ml. measuring flask and made up to the mark with distilled water. After mixing well the extinction is measured in a Zeiss Elko III photometer, filter S.53.E and a 2.0 cm. cuvette used.

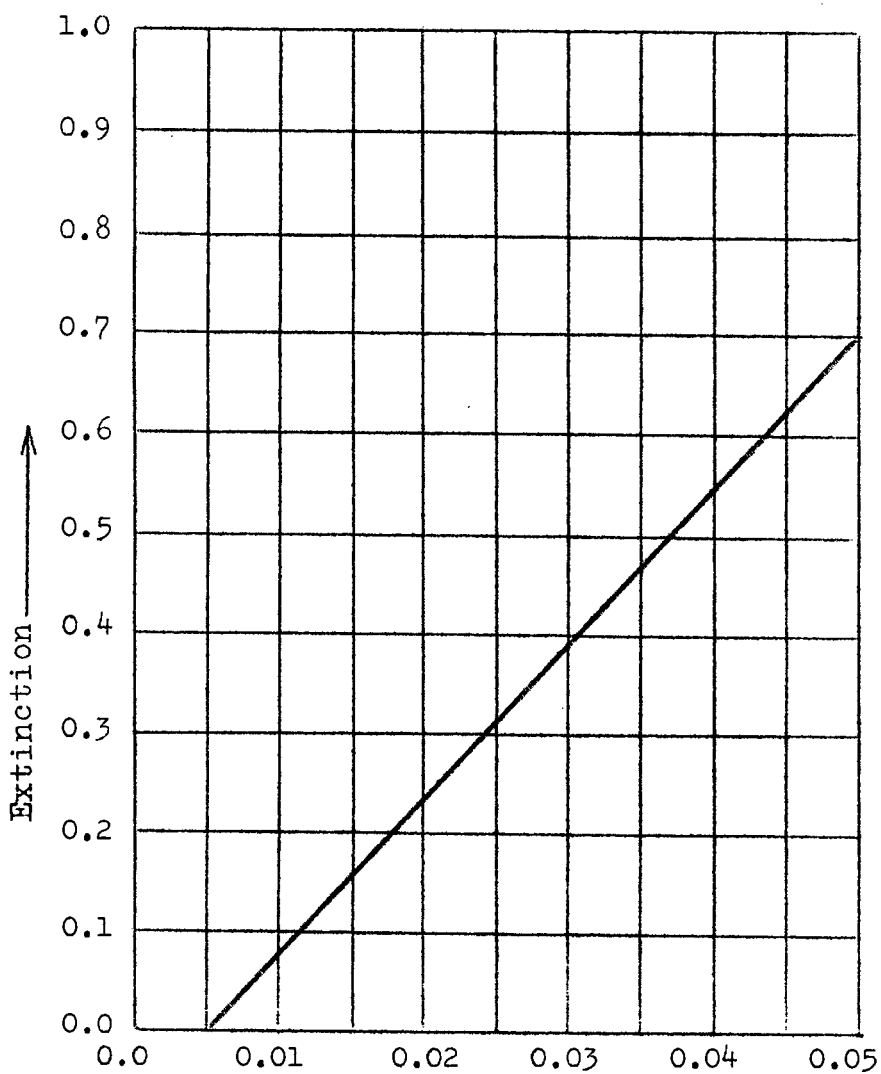
An unstained portion of the paper chromatogram is removed, treated exactly as the nickel-containing sample and used as a reference blank. From a standard calibration curve the amount of nickel (i.e. alcohol) can be read.

Standard Graph.

From a standard solution containing 1 gm. (calculated as ethyl acetate) of the alcohol to be determined per 100 ml. of water, increasing concentrations are pipetted into a 5 ml. solution of 20% sodium hydroxide. Subsequently the procedure as described is followed.

Graph No. 9.

Standard Graph for the
Determination of Ethyl Alcohol.



Ethyl Alcohol → mg
Filter S.57.E
Cuvette 2 cm.

Check on the Method.

When an analysis for methanol or propanol is carried out in the presence of 100 mg. of ethanol the same results are obtained as when no ethanol were present. To a wine which was found to contain ethanol and only traces of amyl alcohol combined in its esters, different quantities of methyl, n-propyl and n-butyl alcohol were added and subsequently analysed according to the method discussed.

TABLE XXI. RECOVERY OF ADDED METHYL, n-PROPYL AND n-BUTYL ALCOHOL (CALCULATED AS ETHYL ALCOHOL)

Alcohol.	Added amount mg/l.	Determined amount mg/l.
Methyl alcohol	5	4.8
	25	24.4
	50	51.2
n-Propyl alcohol	5	4.8
	25	23.7
	50	48.6
n-Butyl alcohol	5	4.7
	25	23.8
	50	48.4

In the above table the average values of ten different analyses are given.

The fact that apart from ethyl alcohol (due to its relatively high concentration in wine) practically no other higher alcohol passes into the pentane extract, can be illustrated as follows:-

The pentane extract of a wine was divided into two equal aliquots. To the one portion sodium hydroxide solution was added and further treated as explained above. To the other portion 5 ml. of distilled water was added and after agitation with the vibrator mixer the pentane was carefully removed by distillation. After cooling 1 gm. of dry sodium hydroxide was dissolved in the water and the xantogenates developed. On comparing the chromatograms of the two pentane aliquots it could be seen that the unhydrolysed extract barring the spot for

ethyl.....

ethyl alcohol yielded no other spot, while the chromatogram for the hydrolysed extract in addition to ethyl alcohol showed the spots for methyl, butyl and amyl alcohols

MICRO-DETERMINATION OF ALDEHYDES AND KETONES.

Reagents:

1. Pentane (E. Merck, Darmstadt).
2. Peroxide free diethyl ether.
3. 5% Sodium hydroxide.
4. 2,4- Dinitrophenylhydrazine solution. This consists of 1.25 gm. 2,4-dinitrophenylhydrazine, 80 ml. concentrated hydrochloric acid and 420 ml. distilled water. The solution is shaken intermittently and filtered after two hours.

Remarks.

A sample of methyl alcohol is rendered free from carbonyl groups by the addition of 2-3 gm. of 2,4-dinitrophenylhydrazine to 250 ml. of methyl alcohol. Furthermore 5-10 ml. of concentrated hydrochloric acid is added. The mixture is well mixed and allowed to stand overnight. The methyl alcohol is distilled off. From this methyl alcohol a quantity of 2,4-dinitrophenylhydrazine is re-crystallised twice.

5. 10% Hydrochloric acid.
6. Pyridine pure (anhydrous).
7. Rhodamine G. 0.02% in methyl alcohol.
8. Ethylenediamine hydrate (E. Merck, Darmstadt).
9. Absolute alcohol.

Solutions for Development.

- I. (a) Methanol saturated heptane.
(b) Heptane saturated methanol.
- II. 95% of a mixture of equal volumes of hexane and heptane and 5% of diethyl ether.

Whatman No. 1. paper.

5% of diethyl ether. The separation is completed once the solvent front has travelled approximately 25 cm. from the starting point.

The chromatograms developed according to the above two procedures are dried at room temperature.

TABLE XXII. Rf-VALUES FOR THE 2,4-DINITROPHENYLHYDRAZONES OF DIFFERENT ALDEHYDES AND KETONES.

2,4-Dinitrophenylhydrazone of	I. Ether-Heptane- Hexane.	II. Methyl alcohol- Heptane.
Formaldehyde	0.81	0.10
Acetaldehyde	0.80	0.21
Propionaldehyde	0.55	0.32
Acetone	0.85	0.31
n-Butylaldehyde	0.82	0.44
Methyl-ethyl ketone	0.88	0.45
Crotonaldehyde	-	0.25
Furfural	0.60	0.05
Benzaldehyde	0.60	-
Salicyl aldehyde	0.38	-
Cinnamic aldehyde	0.34	-
n-Valeraldehyde	-	0.52
2,4-Dinitrophenylhydrazone	0.05	-

The above values represent the averages for ten estimations, the Rf-value for each substance being ascertained five times singly and five times in a mixture of at least four of the above substances.

Quantitative Determination.

A piece of chromatograph paper is sprayed with Rhodamine reagent, dried and used as fluorescent background under ultra violet light.

After the chromatographic separation the dried chromatograms are placed over the fluorescent paper under ultra violet light. The different 2,4-dinitrophenylhydrazones which by daylight present a yellow to yellowish aspect are indicated by dark-bluish spots under ultra violet light and are demarcated

with.....

with a lead pencil. Subsequently the spots are cut out, cut up into small strips and transferred into a 10 ml. measuring flask. For extraction 5 ml. of pyridine is added, the contents of the measuring flask being intermittently shaken for half-an-hour. 0.2 ml. Ethylenediamine hydrate is added, the flask filled to the mark with pyridine and the mixture shaken.

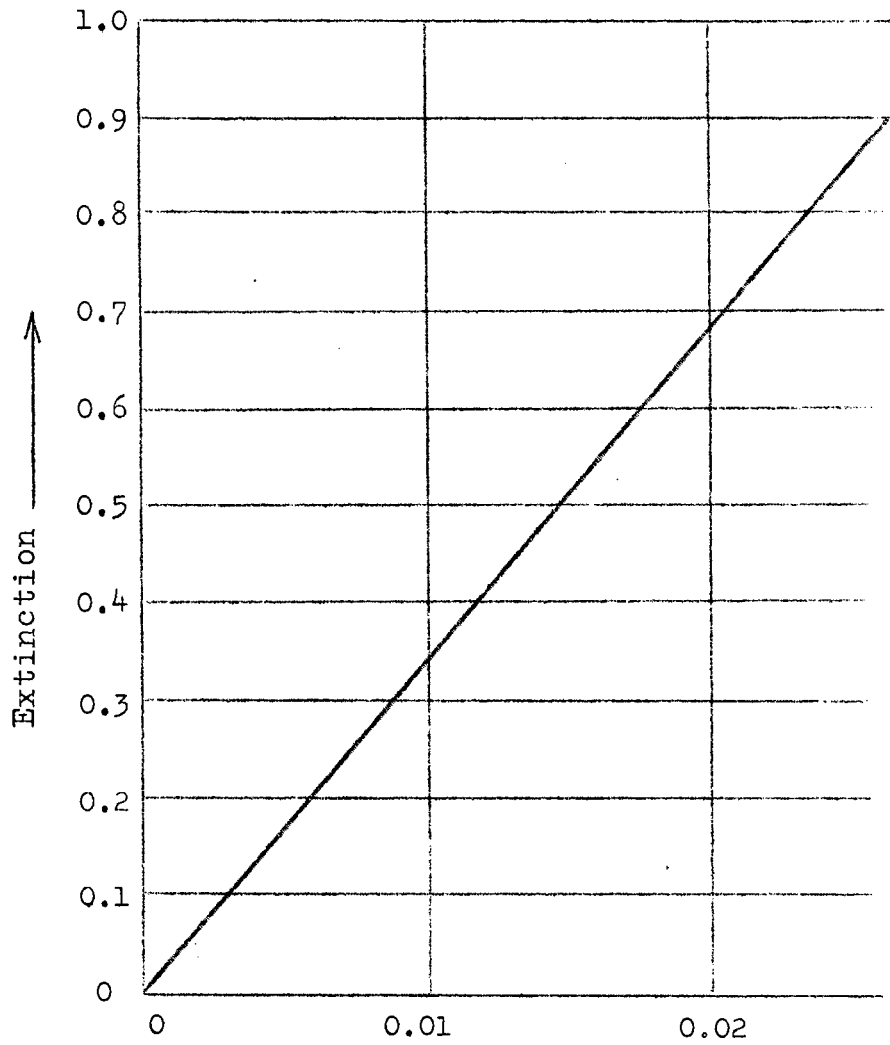
The extinction of the resulting reddish solution is measured in a Zeiss Elko III photometer, using S.53.2 filter and 2.00 cm. cuvettes. The concentration of individual aldehydes and ketones, computed as acetone, can be gained from a standard graph.

Standard Graph.

As a standard solution 1 gm. of the aldehyde or ketone (referred to acetone) is dissolved in 100 ml. of absolute alcohol. From this standard solution, required volumes are transferred by means of an Agla pipette into a pentane : ether (1:1) mixture. This mixture is then treated as described above.

The method has a margin of error of approximately 5% for 10^{-3} to 10^{-5} molecular concentrations of examined aldehydes and ketones. Within this concentration margin, concentration is directly proportional to carbonyl content.

Graph No. 10. Standard Graph
for the Determination of Acetone.



Acetone → mg.

Filter S.53.E

Cuvette 2 cm.

Check on the Method.

The average values for aldehydes and ketones in a wine which was analysed three times were the following:-

Acetaldehyde	67.0 mg/l.
Cinnamic aldehyde	+
Valeraldehyde	2.5 mg/l.

These values were calculated as acetone. To this wine different aldehydes and ketones (likewise referred to as acetone) were added.

TABLE XXIII. THE RECOVERY OF ADDED ALDEHYDES AND KETONES.

Substance added	Amount added (as acetone) mg/l	Amount Determined (as acetone) mg/l
Furfural	10	9.5
	20	19.2
Acetaldehyde	10	8.8 ⁺
	20	17.1 ⁺
Cinnamic aldehyde	10	9.6
	20	19.0
Acetone	10	9.6
	20	18.9
Ethyl-methyl ketone	10	9.4
	20	19.2

⁺Because of the high volatility of the compound, the addition of this substance is difficult to control.

The values represent the averages for ten determinations.

PRACTICAL INVESTIGATIONS.

The first determination of the aroma substances by the uni-dimensional method was carried out on a Geisenheimer Morschberg. This must have had its sugar content increased to 90° Oechsle by "wet" sugaring, i.e. the addition of sugar solution.

The analytical series were the same as that given in Table VII, page 33.

TABLE XXIV. THE FATTY ACIDS (AS COMBINED IN THE ESTERS) FOUND IN A 1956 ORISENHIMER MORSCHBERG.

Analysis: Days after Crushing	Key:						
	1. Formic acid.	2. Acetic acid.	3. Propionic acid	4. Butyric acid	5. Iso-valeric acid	6. Caproic acid	7. Capric acid
	1. Original Clear	2. Original Turbid	3. Panaym Liquid Clear	4. Panaym Liquid Turbid	5. Panaym Solid Clear	6. Panaym Solid Turbid	
0	2 4 5 6	2 4 5 6	2 4 5 6	2 4 5 6	2 4 5 6	2 4 5 6	2 4 5 6
3	2 4 5 6	2 4 5 6	2 4 5 6	2 4 5 6	2 4 5 6	2 4 5 6	2 4 6 6
6	2 3 4 5 6	2 4 5 6	2 3 4 5 6	1 2 4 5 6	2 4 5 6	2 4 5 6	2 4 5 6
10	2 3 4 5 6	2 3 4 5 7	2 3 4 5 6	1 2 4 5 6	2 3 4 5 6	2 3 4 5 6	2 3 4 5 6
13	1 2 3 4 5 6 7 ⁺	2 3 4 5 6 7 ⁺	2 3 4 5 6 7 ⁺	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	2 3 4 5 6
20	1 2 3 4 5 6 7 ⁺	2 3 4 5 6 7	2 3 4 5 6 7 ⁺	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	2 3 4 5 6
32	1 2 3 4 5 6 7 ⁺	2 3 4 5 6 7 ⁺	2 3 4 5 6 7 ⁺	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	2 3 4 5 6

⁺ Contains the aliphatic homologues from and including C₇ upwards, as well as the aromatic fatty acids. The two-dimensional method was not yet developed at this stage.

In the unfermented must three to four acid components of the esters could be detected: acetic, butyric, isovaleric and caproic acids being represented. On the sixth day after crushing the propionic acid could be discerned, whilst on the tenth to the thirteenth day, formic acid could be detected in some instances. Likewise at this stage capric acid and higher homologues and the aromatic fatty acids appeared. On the 32nd day after onset of fermentation up to seven different acid components of the esters, the capric acid being regarded as one, could be found. In three instances no formic acid was present, while in three further instances no higher homologues from C₇ upwards could be found.

In the meantime it had become possible to determine the fatty acids by two-dimensional chromatography. Thus during the 1957 season, analyses of the esters were undertaken from time to time during the ripening stages of musts from six different varieties. The varieties were Riesling, Sylvaner, Ruländer, S.88, Pinot noir (Blauer Burgunder) and Müller-Thurgau.

TABLE XXV

THE QUALITATIVE ANALYSES FOR ESTERIFIED FATTY ACIDS IN SIX
DIFFERENT VARIETIES DURING THE PROGRESSIVE STAGES OF RIPENING.

Varieties	Date of Analysis	°Oechsle in Must	Total Acids gm/l.	Tartaric Acid gm/l.	pH- Value	Esters of Fatty Acids					Esters of Alcohol	Aldehydes and Ketones	
						Formic Acid	Acetic Acid	Propionic Acid	Butyric Acid	Capric [‡] Acid	Ethyl Alcohol	Acet- aldehyde	Valer- aldehyde
Riesling	12. 9.1957	45.8	22.2	10.3	2.62	+	+				+	+	
Sylvaner		56.8	16.2	7.4	2.84	+	+				+		
Ruländer		57.3	17.6	7.5	2.81			+			+		
S.88		46.8	19.8	7.5	2.72	+			+	+	+	+	
Pinot noir		55.8	15.8	5.7	2.86				+		+		
Müller-Thurgau	66.8	11.5	3.8	2.96				+	+	+			
Riesling	18. 9.1957	59.6	20.7	10.8	2.60	+	+		+		+	+	
Sylvaner		64.4	17.3	7.0	2.75	+	+				+	+	
Ruländer		70.8	18.0	7.6	2.80	+	+				+		
S.88		58.6	20.8	8.0	2.68	+	+		+	+	+	+	
Pinot noir		71.6	16.8	6.8	2.83				+		+		
Müller-Thurgau	68.5	12.0	3.8	2.95				+	+	+	+		
Riesling	1.10.1957	60.4	17.1	8.2	2.71	+	+		+		+	+	+
Sylvaner		64.8	14.0	6.9	2.85	+	+				+	+	
Ruländer		74.2	13.2	6.0	2.89	+	+				+	+	
S.88		63.6	17.0	7.8	2.70	+	+		+	+	+	+	
Pinot noir		74.6	15.0	6.9	2.92				+		+	+	
Müller-Thurgau	66.0	10.8	3.7	3.00				+	+	+	+		
Riesling	11.10.1957	67.0	16.0	5.7	2.89	+	+		+	+	+	+	+
Sylvaner		72.0	12.9	2.8	3.10	+	+				+	+	
Ruländer		79.5	12.2	4.8	3.15	+	+				+	+	
S.88		72.0	15.7	6.6	2.93	+	+		+	+	+	+	
Pinot noir		-											
Müller-Thurgau	-												
Riesling	22.10.1957	62.0	13.1	4.2	3.06	+	+	+	+	+	+	+	+
Sylvaner		66.2	11.3	4.1	3.20	+	+				+	+	
Ruländer		76.6	11.5	2.6	3.24	+	+				+	+	
S.88		79.6	13.0	2.6	3.05	+	+	+	+	+	+	+	
Pinot noir		-											
Müller-Thurgau	-												

‡ Denotes all higher homologues from C₇ upwards and aromatic fatty acids.

In the Riesling must the esters consisted of the ethyl alcohol esters of formic and acetic acids. Acetaldehyde was already present in unripe grapes. Later propionic and capric acids and traces of valeraldehyde became detectable. With Sylvaner only the ethyl esters of formic and acetic acids could be found in the beginning. Later acetaldehyde made its appearance. The same picture was represented by Ruländer and Pinot noir.

Vastly different from the above were the varieties S.88 and Müller-Thurgau. The S.88, even at a comparatively unripe stage, showed the presence of the ethyl esters of formic, acetic, butyric, capric and higher acids. The only aldehyde found to be present was acetaldehyde. The Müller-Thurgau grapes apart from the absence of formic acid presented the same picture as the S.88 grapes immediately prior to harvesting. It is a recognised fact that both these varieties yield wines rich in bouquet. According to the above data it must be assumed that a large proportion of such aroma substances is formed in the berry.

Riesling wines from the same vineyards but different vintages were analysed to establish possible differences in the composition of their volatile substances.

In addition to the total analysis (Table XXVI) the esterified acids and alcohols as well as the aldehydes and ketones were determined (Table XXVII). In this last-mentioned table the acids and alcohols were calculated as ethyl acetate and the aldehydes and ketones as acetone.

TABLE XXVI

TOTAL ANALYSIS OF GEISENHEIMER RIESLING FOR DIFFERENT VINTAGES.

	1951 Vat 59	1951 Vat 67	1951 Vat 71	1951 Vat 72	1954 Vat 38	1954 Vat 41	1954 Vat 42	1953 Vats 28/29	1953 Vat 56
Date of Crushing	23.11.1951	26.11.1951	27.11.1951	27.11.1951	27.10.1954	27.10.1954	27.10.1954	20.10.1954	20.10.53
Sugar Content °Oechsle	77	83	81.5	81.5	63.4	63.4	63.4	105.1	91.9
Improved or natural	Sugar solid added to 95°	natural	natural	natural	Sugar solid added to 98°	natural	Sugar solid added to 98°	natural	natural
Acid content	10.5	11.7 (neutralised with CaCO ₃ to 10.7)	9.6	9.6	15.0 (neutralised with CaCO ₃ to 12.0)	15.0	15.0	9.2	9.0
Specific Gravity	1.0026	0.9975	0.9968	0.9967	0.9945	1.0003	0.9967	0.9999	0.9990
Alcohol gm/l.	83.8	84.5	80.4	85.1	91.9	60.6	95.4	104.3	93.3
Volume %	10.61	10.70	10.18	10.78	11.64	7.68	12.08	13.21	11.82
Total Extract gm/l.	43.1	30.0	26.8	28.1	23.8	27.6	32.0	43.4	37.5
Sugar-free Extract gm/l.	26.8	27.2	24.5	25.2	22.6	26.8	27.0	28.1	28.7
Sugar gm/l.	17.3	3.8	3.3	3.9	2.5	1.8	6.0	16.3	9.8
Glycerine gm/l.	8.7	-	-	-	-	-	-	-	-
Total Acidity gm/l.	8.8	9.5	8.4	8.5	6.5	10.5	9.5	7.0	7.0
Tartaric Acid gm/l.	7.3	2.1	3.1	2.9	1.5	3.3	2.7	1.3	1.8
Lactic Acid gm/l.	0.5	0.5	-	-	-	-	-	-	-
Volatile Acid gm/l.	0.3	0.4	0.4	0.4	0.5	0.3	0.4	0.5	0.6
Ash gm/l.	2.02	2.12	1.87	1.93	2.27	2.22	1.92	2.68	2.20
Ash Alkalinity (ml. of N. NaOH/Liter)	17.7	18.0	20.3	20.3	-	-	-	20.0	20.0

TABLE XXVII

THE ANALYSIS OF THE VOLATILE CONSTITUENTS OF DIFFERENT VINTAGES
OF GEISENHEIMER RIESLING.

Acids mg/l.	1951 Vat 59	1951 Vat 67	1951 Vat 71	1951 Vat 72	1954 Vat 38	1954 Vat 41	1954 Vat 42	1953 Vats 28/29	1953 Vat 56
Formic acid	15.6	8.4	26.5	4.6	8.6	12.3	8.3	26.0	22.6
Acetic acid	66.0	58.4	46.2	63.0	48.5	43.3	70.4	56.9	63.5
Propionic acid	+				+			+	+
Butyric acid	6.3	10.3	4.4	3.0	18.3	6.6	14.7	16.0	7.9
Iso-valeric acid	4.2			3.4		6.4	+	+	
Caproic acid		9.9	+		+		3.2	6.4	+
Capric acid								12.8	+
Cinnamic acid								4.8	1.2
Benzoic acid								+	
	92.1	87.0	77.1	74.0	75.4	68.6	96.6	112.9	95.2
<u>Alcohols mg/l.</u>									
Methyl alcohol	12.8	2.1	4.0	+		+	3.5	6.4	2.7
Ethyl alcohol	68.3	68.6	64.8	65.6	64.1	68.6	90.7	90.1	80.2
Propyl alcohol								+	+
Butyl alcohol									+
Amyl alcohol	11.0	16.3	8.3	8.4	11.3		2.4	16.4	12.3
	92.1	87.0	77.1	74.0	75.4	68.6	96.6	112.9	95.2
<u>Aldehydes and Ketones mg/l.</u>									
Formaldehyde	4.3	5.6	6.3	+	+	+	6.3	4.2	+
Acetaldehyde	75.4	53.7	65.0	68.5	58.6	80.9	74.6	77.4	68.5
Propionaldehyde								+	
Valeraldehyde	1.7	6.7	7.8	2.4	+		6.3	6.3	6.8
Acetone	2.4	+		1.6	+		+	+	+
Ethyl-methyl ketone								+	+
Cinnamic aldehyde							+	2.8	+
Benzaldehyde								0.9	
	83.8	66.0	79.1	72.5	58.6	80.9	87.2	86.6	77.1

These investigations yielded the following interesting picture. The higher the sugar content of the original must, the more esters are afterwards found in the wine. Also musts which had been improved artificially by the addition of sugar showed an increased ester content by comparison to wines resulting from the same original but unsugared must.

In the recognised vintages like for instance 1953, the higher homologues and aromatic substances figure in increasing concentrations. The concentration of aldehydes and ketones move more or less parallel to that of the esters. Better vintages and wine from improved musts show higher aldehyde and ketone contents. Cinnamic and benzaldehydes were not detectable in wines from improved musts, while they were strongly represented in the good vintages, i.e. in wines originating from musts with high sugar contents. The aroma of the vintage wines must be ascribed to the higher and aromatic homologues of the fatty acids combined in the esters, as well as the higher content of aldehydes and ketones.

The next logical step was to try and establish which esters, aldehydes and ketones are formed when a sugar (artificial must) solution containing no such original compounds is fermented. To 2,500 ml. of an artificial must showing the following analysis, 100 and 150 gm/l. sugar were respectively added:-

Tartaric acid	4 gm/l
Malic acid	4 "
Tannin	0.3 "
Yeast autoysate	1 "
Sec. Ammonium phosphate	2 "
Mono-potassium phosphate	2 "
Magnesium sulphate	1 "

After the above substances were dissolved in tap water the samples were filtered through an E.K. filter.

Samples immediately withdrawn showed that no esters, aldehydes or ketones were present. After completed fermentation the two artificial musts were analysed for esters, i.e. acid and alcohol components and aldehydes and ketones.

In the sample which originally contained 100 gm./l. of sugar, 36 mg/l. of acetic acid and 43.5 gm/l. of acetaldehyde were found. Apart from ethyl alcohol no other alcohols could be detected. In the sample which originally contained 150 gm/l. of sugar, 53.1 mg/l. of acetic acid and 77.2 mg/l. of acetaldehyde could be found. Formaldehyde was present in traces only. In addition to ethyl alcohol no other alcohols could be detected.

An important observation which could bear further examination was the fact that in white hybrid wines substances could be detected the presence of which could not in the course of this investigation be indicated in wines from pure *Vitis vinifera* grapes. In one hybrid wine phthalic acid could be found and in the same sample in addition to two other samples from hybrids, anthranilic acid was present.

With further experience it is conceivable that these substances could form a possible basis for the detection of white hybrid wines or blends of *vinifera* and hybrid wines.

In conclusion two brandy samples were analysed for their ester content. The one brandy was a German and the other a French product.

The brandy samples were diluted to three times their original volume and otherwise treated exactly as explained under the various methods of analyses.

The total esters were first determined according to the method of Hennig¹⁰⁵⁾. The German brandy and the French brandy showed a total ester content of 366 mg/l. and 388 mg/l. respectively. The acid and alcohol fractions of the esters were determined and calculated according to the discussed method. The results of these determinations are given in the following table.

TABLE XXVIII

ANALYSIS OF TWO BRANDY SAMPLES.

Estimation of Fatty Acid Fractions.

	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇
Brandy	Formic Acid mg/l.	Acetic Acid mg/l.	Propionic Acid mg/l.	Butyric Acid mg/l.	Valeric Acid mg/l.	Caproic Acid mg/l.	Enanthic Acid mg/l.
German Brandy	28.0	172.0		22.0		44.0	-
French Brandy	17.6	240.0	-	-	-		25.0
Calculated as ethyl acetate with the factor:							
Factor	1.7	1.47	1.26	1.0	0.87	0.75	0.5
German Brandy	47.6	258.0		24.0		35.0	-
French Brandy	29.9	353.0	-	-	-		15.5

The above values together leave the German and French Brandy with 364.6 and 398.0 mg/l. ester as ethyl acetate respectively.

Estimation of the Alcohol Fractions.

	C ₁	C ₂	C ₃	C ₄	C ₅
Brandy	Methyl alcohol mg/l.	Ethyl alcohol mg/l.	Propyl alcohol mg/l.	Butyl alcohol mg/l.	Amyl alcohol mg/l.
German Brandy	24.0	115.0 ⁺	-	-	79.0
French Brandy	-	161.0 ⁺	-	-	90.0
Calculated as ethyl acetate with the factor:					
Factor	2.75	1.91	-	-	1.0
German Brandy	66.0	219.6 ⁺	-	-	79.0
French Brandy	-	308.0 ⁺	-	-	90.0

+ Values calculated by difference

S U M M A R Y.

A qualitative and quantitative micro-analytical method for the determination of the esters is proposed. For the estimation of the esterified carbonic acids a two-dimensional method is worked out. The Rf-values of the hydroxamic acids of the different fatty acids examined are furnished. Standard reference graphs for acetic, phthalic, cinnamic and anthranilic acids are supplied.

A quantitative method of analysis for the esterified alcohols is also proposed. The Rf-values for the xantogenates of the different alcohols as well as a standard reference graph are given.

The aldehydes and ketones are also quantitatively determined. A standard reference graph for all examined aldehydes and ketones (calculated as acetone) is furnished.

With the vibrator mixer it was found possible to carry out a hydrolysis with a solution of sodium hydroxide added to an immiscible solution, e.g. pentane or ether. The apparatus allows an intensive mixing in conjunction with controlled heating.

The intensive mixing and good shut-off, considerably aided the quantitative determination of the bouquet substances.

Unfermented musts already contained from three to four esterified acids, acetic, butyric, isovaleric and caproic acids being present. During fermentation the esters increase. After fermentation up to seven different esterified fatty acids were observed.

The quantitative determination of the aroma substances proved that musts from different varieties contained different esters. The varieties S.88 and Müller Thurgau contained the ethyl esters of acetic, butyric, capric and aromatic acids, the former also containing formic acetate.

The examination of wines from different vintages proved that the higher the original sugar content of the must the more esters are liable to be found in the ultimate wine. Better vintages as well as wines from improved musts show

higher.....

higher contents of aldehydes and ketones. In wines from improved musts cinnamic aldehyde and benzaldehyde could not be found. The bouquet of vintage wines must be ascribed to their higher ester and aldehyde and ketone content.

Fermented sugar solutions, apart from acetaldehyde, showed only ethyl acetate to be present.

The observation that white hybrids can contain phthalic and anthranilic acids, the presence of which remained undetectable in wines from European (vinifera) varieties, indicates possibilities for the identification of such hybrid wines.

In conclusion an estimation of the esters of two samples of brandy was carried out.

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A P P E N D I X II.

AROMA SUBSTANCES.

Fig. 1.

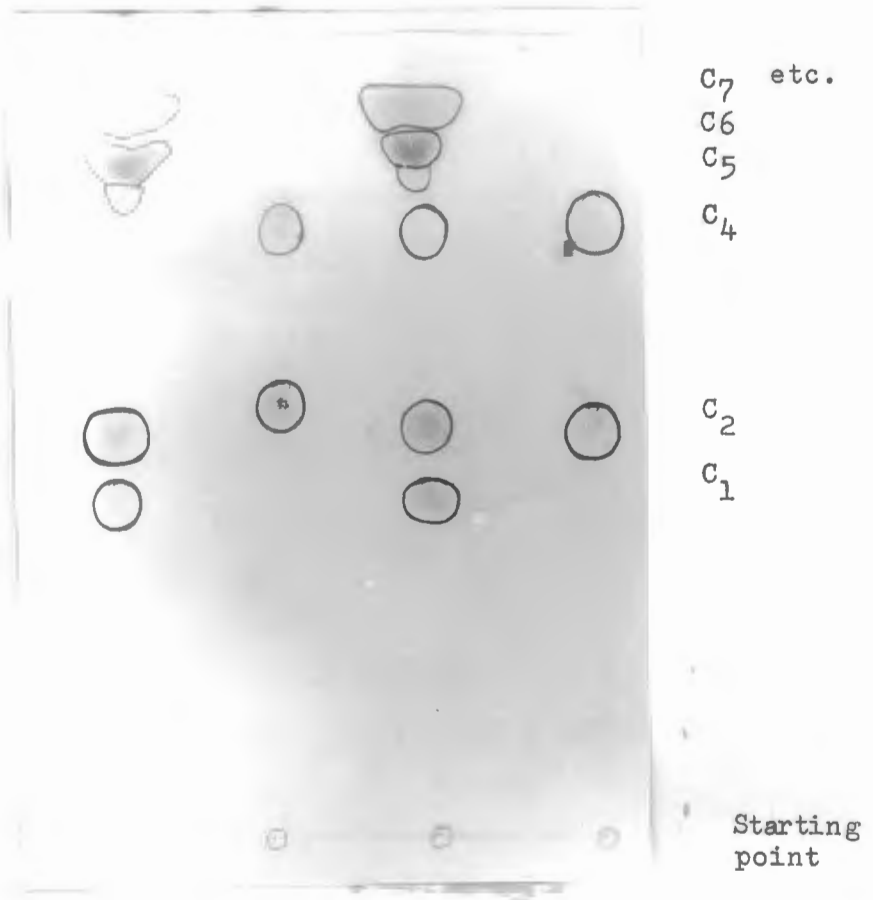
Chromatogram of Hydroxamic Acids.

Solvent: Dimethyl formamide - n-Butanol - Water.

Developing Time: 16 Hours (Downwards).

Spray: Ferric Chloride.

Paper: S. & S. 2043b.



Wine

Wine

Reference
solution

Reference
solution

Fig. 2.

Chromatogram of Hydroxamic Acids.

Solvent: Tetrahydro furfurane - Ethyl acetate - water.

Developing Time: 10 Hours (Upwards).

Spray: Ferric Chloride.

Paper: S. & S. 2043b acetylated.

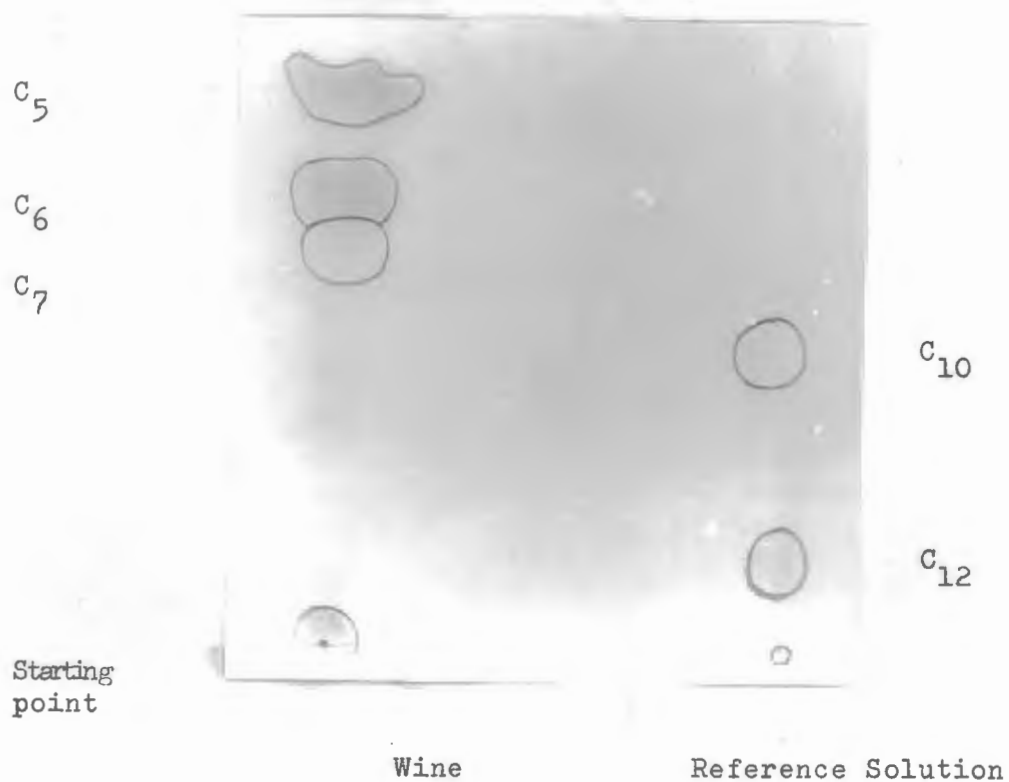


Fig. 3.

Chromatogram of the Xantogenates of
Lower Saturated Alcohols.

Developing time: 6 Hours (upwards).

Solvent: Water-saturated n-Butanol.

Spray: 10% Ni SO₄ solution.

Paper: S. & S. 2043b (impregnated with Phosphate
Buffer pH 12.0).

